



Diet, immunity and inflammation

Edited by Philip C. Calder and Parveen Yaqoob

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Diet, immunity and inflammation

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**Edited by
Philip C. Calder and Parveen Yaqoob**



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Contents

<i>Contributor contact details</i>	xv
<i>Woodhead Publishing Series in Food Science, Technology and Nutrition</i>	xxi

Part I Immunity and inflammation: an introduction 1

1 Innate and adaptive immunity 3

P. S. Noakes, University of Western Australia, Australia and L. J. Michaelis, Royal Victoria Infirmary, UK

1.1 Introduction	3
1.2 Levels of defense	4
1.3 Innate immunity	4
1.4 Adaptive immunity	19
1.5 Conclusion	29
1.6 References	30

2 Mucosal immunity in a healthy gut 34

P. Brandtzaeg, University of Oslo, Norway

2.1 Introduction	34
2.2 Induction of adaptive immunity in the gut	37
2.3 Secretory immunity reinforcing the mucosal barrier	44
2.4 Immunological consequences of gut microbial–host interactions	51
2.5 IgA and breastfeeding in immune control	55
2.6 Induction of homeostasis <i>versus</i> hypersensitivity in the gut	57
2.7 Conclusion	64
2.8 Acknowledgments	65
2.9 References	66

3 Chronic inflammatory diseases 81

A. Kiliç, D. A. Kesper, P. I. Pfefferle and H. Renz, Philipps-University at Marburg, Germany

3.1 Introduction	81
3.2 Epidemiological trends of chronic inflammatory diseases	83
3.3 T-helper cells: differentiation and effector cell plasticity	83
3.4 Chronic inflammation and epigenetics	87
3.5 MicroRNA: biogenesis, function and role in regulating adaptive immunity	94

FOR REFERENCE PURPOSES ONLY

vi Contents

3.6	Conclusion and future trends	99
3.7	References	100
4	Chronic low-grade inflammation	105
	<i>A. N. Margioris, E. Dermizaki, M. Venihaki and C. Tsatsanis, University of Crete, Greece</i>	
4.1	Introduction	105
4.2	Obesity and the development of chronic low-grade inflammation (CLGI)	106
4.3	Adipose tissue as an immune organ	108
4.4	White and brown adipose tissue and CLGI	109
4.5	Adipocytes as immune cells	110
4.6	Impact on inflammation of other cells from adipose tissue	113
4.7	The anti-inflammatory status of lean adipose tissue	116
4.8	Laboratory evaluation of CLGI	116
4.9	References	117
Part II	Micronutrients, immunity and inflammation	121
5	The role of zinc in immunity and inflammation	123
	<i>K. Gruber and L. Rink, RWTH-Aachen University Hospital, Germany</i>	
5.1	Introduction	123
5.2	Nutritional background	124
5.3	Molecular mechanisms of zinc action	128
5.4	Effects of zinc on immune function	132
5.5	The impact of zinc in inflammatory processes	137
5.6	Zinc in human health and clinical nutrition	138
5.7	Conclusion and future trends	145
5.8	References	146
6	The role of dietary selenium in inflammation and immunity	157
	<i>Z. Huang, Jinan University, P. R. China and P. R. Hoffmann, University of Hawaii, USA</i>	
6.1	Introduction	157
6.2	Nutritional aspects of selenium	158
6.3	The selenoprotein family	162
6.4	The effect of decreased selenium (Se) intake on selenoprotein synthesis	165
6.5	Selenium and viral immunity	166
6.6	Selenium and bacterial and fungal infections	168
6.7	Selenium and immunity against parasites	170
6.8	Selenium and inflammation related to critical illness, clinical sepsis and colitis	172
6.9	Selenium and allergies and asthma	174
6.10	Selenium, cancer and immunity in aging	176

6.11	Mechanisms by which dietary selenium may affect	
	inflammation and immune responses	178
6.12	Conclusion and future trends	180
6.13	Sources of further information and advice	182
6.14	References	182
7	The role of iron in immunity and inflammation: implications for the response to infection	193
	<i>S. R. Kuvibidila, United Arab Emirates University, United Arab Emirates (formerly of Oklahoma State University, USA), S. B. Baliga, University of South Alabama, USA, L. C. Chandra, Tulane University, USA and C. L. French, Children's Hospital at Harvard University, USA</i>	
7.1	Introduction	193
7.2	The effects of iron deficiency on immunity	201
7.3	The effects of iron overload on immunity	207
7.4	Mechanisms of impaired immunity in iron deficiency	209
7.5	Iron status and infection	211
7.6	Conclusion	213
7.7	Acknowledgments	213
7.8	References	214
8	Vitamin A and the immune system	221
	<i>A. C. Ross and K. H. Restori, Pennsylvania State University, USA</i>	
8.1	Introduction	221
8.2	Sources, forms and dietary recommendations for vitamin A . . .	222
8.3	Major aspects of normal vitamin A metabolism	223
8.4	Influence of inflammation on vitamin A transport and metabolism	226
8.5	Pro-inflammatory and anti-inflammatory aspects of retinoids in the skin	228
8.6	Vitamin A and immunity	230
8.7	Future trends	237
8.8	Acknowledgments	237
8.9	References	237
9	Vitamin D and the immune system	244
	<i>N. C. Harvey, MRC Lifecourse Epidemiology Unit, University of Southampton, UK and M. T. Cantorna, Center for Molecular Immunology and Infectious Disease, The Pennsylvania State University, USA</i>	
9.1	Introduction	244
9.2	Food sources, recommended intakes and epidemiology of deficiency	248
9.3	Disease states associated with vitamin D	250

FOR REFERENCE PURPOSES ONLY

viii Contents

9.4	Vitamin D and the immune system	251
9.5	Predictions and state of the evidence to support vitamin D interventions to alter immune function	257
9.6	Conclusion	257
9.7	References	258
Part III Other dietary constituents, immunity and inflammation . . .		265
10 Intestinal bacteria and probiotics: effects on the immune system and impacts on human health		267
<i>F. Trejo and Y. Sanz, Instituto de Agroquímica y Tecnología de Alimentos Consejo Superior de Investigaciones Científicas (IATA-CSIC), Spain</i>		
10.1	Introduction	267
10.2	Mechanisms of action of intestinal bacteria and probiotics on the immune system	269
10.3	Evidence of the efficacy of probiotics in animal models	274
10.4	Evidence of the efficacy of probiotics in humans	278
10.5	Conclusion	283
10.6	Acknowledgements	283
10.7	References	283
11 Impacts of prebiotics on the immune system and inflammation . . .		292
<i>F. Guarner, University Hospital Vall d'Hebron, Spain</i>		
11.1	Introduction	292
11.2	Natural sources and prebiotic products	294
11.3	Prebiotic effects in the gut: fermentation and physiology	296
11.4	Prebiotic effects and the immune system	300
11.5	Intestinal inflammation and the metabolic syndrome	302
11.6	The role of prebiotics in human health and clinical nutrition . . .	306
11.7	References	308
12 Food bioactive proteins and peptides: antimicrobial, immuno- modulatory and anti-inflammatory effects		313
<i>P. J. Moughan and K. Rutherfurd-Markwick, Massey University, New Zealand</i>		
12.1	Introduction	313
12.2	Sources of food-derived bioactive peptides and proteins	314
12.3	Physiological effects of food-derived proteins and peptides . . .	317
12.4	Stability of bioactive peptides in the digestive tract, the potential for absorption and site of action	318
12.5	Antimicrobial effects	321
12.6	Effects on immune function	324
12.7	Effects on inflammation	327
12.8	Mechanisms of action	328
12.9	Applications in human health and clinical nutrition	330
12.10	References	333

13	Gangliosides, immunity, infection and inflammation	341
	<i>R. Rueda, Abbott Nutrition, Spain</i>	
13.1	Introduction	341
13.2	Definition and description of gangliosides	342
13.3	Sources and intakes of gangliosides	344
13.4	Mechanisms of action of gangliosides	346
13.5	Effects of gangliosides on immune function	349
13.6	Effects of gangliosides on inflammation	351
13.7	Conclusion and future trends	352
13.8	Acknowledgments	354
13.9	References	354
14	Plant foods and inflammatory processes	359
	<i>B. Watzl, Max-Rubner-Institut, Federal Research Institute of Nutrition and Food, Germany</i>	
14.1	Introduction	359
14.2	Whole grain and refined grains	360
14.3	Dietary fibre	362
14.4	Vegetables and fruits	363
14.5	Soy	367
14.6	Nuts	368
14.7	Coffee and tea	368
14.8	Mechanisms contributing to the anti-inflammatory effects of plant foods	369
14.9	Conclusion	371
14.10	References	371
15	Flavonoids and immune function	379
	<i>F. Ioannone, C. Miglio, A. Raguzzini and M. Serafini, CRA-NUT Research Center for Food and Nutrition, Italy and G. Morabito, IRCCS-San Raffaele Pisana-Food and Nutrition Unit, Italy</i>	
15.1	Introduction	379
15.2	Structure, classification and sources of flavonoids	380
15.3	Bioavailability and metabolism of flavonoids	385
15.4	<i>In vitro</i> effects of flavonoids on cellular functions	388
15.5	Molecular mechanisms	394
15.6	Human intervention studies with flavonoids	397
15.7	Conclusion	404
15.8	Future trends	406
15.9	References	406
16	Immunomodulatory effects of mushroom β-glucans	416
	<i>H. F. J. Savelkoul, Wageningen University, The Netherlands and W. Chanput and H. J. Wichers, Wageningen University and Research Centre, The Netherlands</i>	
16.1	Introduction	416

FOR REFERENCE PURPOSES ONLY

x Contents

16.2	Introduction to β -glucans	417
16.3	Immunological activity of mushroom β -glucans	420
16.4	Immunomodulation by mushroom β -glucans as dietary components	423
16.5	Safety of dietary β -glucans	427
16.6	Conclusion and future trends	428
16.7	References	430
17	Modulation of inflammatory and immune responses by short-chain fatty acids	435
	<i>M. A. R. Vinolo, H. G. Rodrigues, R. T. Nachbar and R. Curi, University of São Paulo, Brazil</i>	
17.1	Introduction: physiology of short-chain fatty acids (SCFAs) . . .	435
17.2	Short-chain fatty acids mechanisms of action	437
17.3	Effect of short-chain fatty acids on leukocyte function	438
17.4	Effect of short-chain fatty acids on inflammation	443
17.5	Perspectives of short-chain fatty acids and inflammation	448
17.6	Sources of further information	448
17.7	Acknowledgement	449
17.8	References	449
18	Long-chain fatty acids and inflammatory processes	457
	<i>P. C. Calder, University of Southampton, UK</i>	
18.1	Introduction	457
18.2	Importance of fatty acid composition for function of cells involved in inflammation	461
18.3	Lipid mediators produced from n-6 and n-3 fatty acids	462
18.4	Effects of specific fatty acids on inflammation	465
18.5	Conclusion	473
18.6	References	475
19	Olive oil and immune function	484
	<i>P. Yaqoob, University of Reading, UK</i>	
19.1	Introduction	484
19.2	The effects of olive oil on various parameters of immune function	485
19.3	Olive oil-containing emulsions in clinical nutrition	492
19.4	Olive oil and inflammatory disease	493
19.5	The contribution of non-lipid components to the immunomodulatory effects of olive oil	495
19.6	Conclusion	497
19.7	References	497
20	The cell- and immune-modulating properties of glutamine	502
	<i>E. Roth, Medical University of Vienna, Austria</i>	
20.1	Introduction	502

20.2	Chemistry and physiology	503
20.3	Immune modulation	504
20.4	Cell modulation	507
20.5	Influence of glutamine on cell signalling and gene expression	510
20.6	Clinical studies with glutamine	510
20.7	Future trends	514
20.8	Conclusion	516
20.9	Sources of further information	516
20.10	References	517
21	Arginine and immune function	523
	<i>G. Wu, Texas A&M University, USA</i>	
21.1	Introduction	523
21.2	Sources of arginine in the circulation	524
21.3	Utilization of arginine by multiple pathways	528
21.4	Roles of arginine in immune responses	531
21.5	Mechanisms by which arginine modulates immune function	534
21.6	Chemical forms and safety of arginine supplementation	536
21.7	Conclusion and future trends	537
21.8	Acknowledgments	538
21.9	References	538
22	Sulphur amino acids and immune functions	544
	<i>G. Grimble, University College London, UK</i>	
22.1	Introduction	544
22.2	Methionine metabolism	548
22.3	Cysteine synthesis and metabolism	548
22.4	S-adenosylmethionine metabolism	550
22.5	Sulphur amino acid requirements following injury or infection	553
22.6	Is supplementation with methionine and cysteine alone the best way forward?	553
22.7	Paracetamol (acetaminophen) overdose as a paradigm for sulphur amino acid supplementation	554
22.8	S-adenosylmethionine and infection	555
22.9	Sulphur amino acids and their effect on illnesses	556
22.10	Conclusion and future trends	559
22.11	References	560
Part IV	Nutrition, immunity and inflammation	571
23	Adverse immune reactions to foods	573
	<i>E. A. Miles, University of Southampton, UK</i>	
23.1	Introduction	573
23.2	Food allergy classification: IgE-mediated, non-IgE-mediated, and mixed IgE- and non-IgE-mediated food allergy	576

FOR REFERENCE PURPOSES ONLY

xii Contents

23.3	IgE-mediated food allergy	577
23.4	Non-IgE-mediated and mixed- or IgE- and non-IgE-mediated food allergy	588
23.5	Coeliac disease	594
23.6	Conclusion	605
23.7	References	606
24	Early nutritional programming	612
	<i>S. E. Moore, MRC International Nutrition Group, UK</i>	
24.1	Introduction	612
24.2	Developmental origins of health and disease	613
24.3	Programming of infectious disease mortality	615
24.4	Programming infant immune development	617
24.5	Programming long-term immune functions	623
24.6	Future trends	626
24.7	References	627
25	The immune system during ageing	631
	<i>L. Müller, Max Planck Institute for Human Development, Germany and G. Pawelec and E. Derhovanessian, University of Tübingen, Germany</i>	
25.1	The components of the immune system and their origins: innate and adaptive, cellular and humoral	631
25.2	The effects of age on the different components of the immune system	636
25.3	Reasons for the observed effects of age on immunity	642
25.4	Conclusion	645
25.5	Future trends	646
25.6	Sources of further information and advice	647
25.7	References	648
26	Exercise, nutrition and immunity	652
	<i>M. Gleeson, Loughborough University, UK</i>	
26.1	Introduction	652
26.2	Effects of exercise on the immune system	653
26.3	Nutritional practices of athletes influencing immune functions	656
26.4	Nutrition strategies to limit exercise-induced immune depression	658
26.5	The evidence for nutritional countermeasures to exercise- induced immunodepression	662
26.6	Conclusions and recommendations	677
26.7	Future trends	677
26.8	Sources of further information and advice	678
26.9	References	679

27 Malnutrition, immunity and infection	686
<i>N. W. Solomons, CeSSIAM in Guatemala, Guatemala</i>	
27.1 Introduction	686
27.2 The interaction of nutrition, infection and immunity	687
27.3 Growth, body composition and infection	690
27.4 Selected micronutrients	694
27.5 Multiple nutritional factors	704
27.6 Newer paradigms on the research horizon	704
27.7 Conclusion	708
27.8 References	709
 <i>Index</i>	 719

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Part I

Immunity and inflammation: an introduction

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1

Innate and adaptive immunity

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Abstract: The innate and adaptive immune systems are often described as contrasting separate arms of the host response; however, they usually act together, with the innate response representing the first line of host defense and the adaptive response becoming prominent after several days as antigen-specific T and B cells have undergone clonal expansion. Components of the innate system contribute to activation of the antigen-specific cells. Additionally, the antigen-specific cells amplify their responses by recruiting innate effector mechanisms to bring about the complete control of invading microbes. Thus, although the innate and adaptive immune responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact, fully effective immune response.

Key words: innate immunity, adaptive immunity, B cell, T cell, regulation.

1.1 Introduction

The immune system consists of a complex network of specialized cells which each have precise roles. Immunity is generally divided into two parts determined by the speed and specificity of the response: (i) innate and (ii) adaptive immunity. The innate immune response is rapid, non-specific, non-anticipatory and non-clonal, and utilizes germ-line-encoded receptors. In contrast, the adaptive (sometimes referred to as acquired) immune response is specific, anticipatory and clonal and uses receptors that have been generated by somatic DNA rearrangement. The innate response uses an arsenal of cells, which includes phagocytic cells (neutrophils, monocytes and macrophages), inflammatory cells (basophils, mast cells and eosinophils) and natural killer cells. The molecular components of the innate repertoire include complement, acute-phase proteins and cytokines. The

adaptive response involves the binding of antigen to surface receptors of specific B and T cells via an antigen presenting cell (APC) and the subsequent activation, proliferation and regulation of many immune pathways. In response to this cascade, B cells secrete immunoglobulins (Ig), the antigen-specific antibodies whose key role is to identify and aid destruction of extracellular micro-organisms. T cells assist B cells to produce antibody and are able to eradicate intracellular organisms by triggering macrophages and directly destroying virally infected cells. Innate and adaptive immune responses work together to eliminate organisms, toxins and allergens, while providing normal homeostasis. This chapter will provide an overview of the key mechanisms used by the immune system to respond to invading microbes and other exogenous threats.

1.2 Levels of defense

In order to protect itself from a diverse array of environmental pathogens and toxins, the body has developed a number of barrier mechanisms (e.g. the gut immune barrier and blood–brain barrier) to limit the entry of potential hazards. Although each barrier provides defense in very different environments, there are many similarities in their mechanisms of action. There is a physical barrier formed by a cellular layer that tightly regulates the movement of ions, molecules and cells between two tissue spaces. These barrier cells interact with different cell types, which dynamically regulate their function, and with a different array of immune cells that survey the physical barrier and provide innate and adaptive immunity (for a recent review of these chemical and physical barriers see (Daneman and Rescigno, 2009)).

1.3 Innate immunity

The innate immune system comprises several molecules, cells and processes that work in conjunction with the adaptive immune system to maintain physiological homeostasis of the host and to protect it against potentially pathogenic organisms. Although innate and adaptive immune systems have been traditionally viewed as two separate entities or sometimes as two overlapping entities, it has now become very clear that these are highly integrated and interconnected systems.

1.3.1 Innate immune recognition

Pattern-recognition receptors

Recognition of microbial pathogens is an essential element for the initiation of innate immune response such as inflammation and is mediated by germline-encoded pattern-recognition receptors (PRRs) that recognize molecular structures that are broadly shared by pathogens, known as pathogen-associated molecular

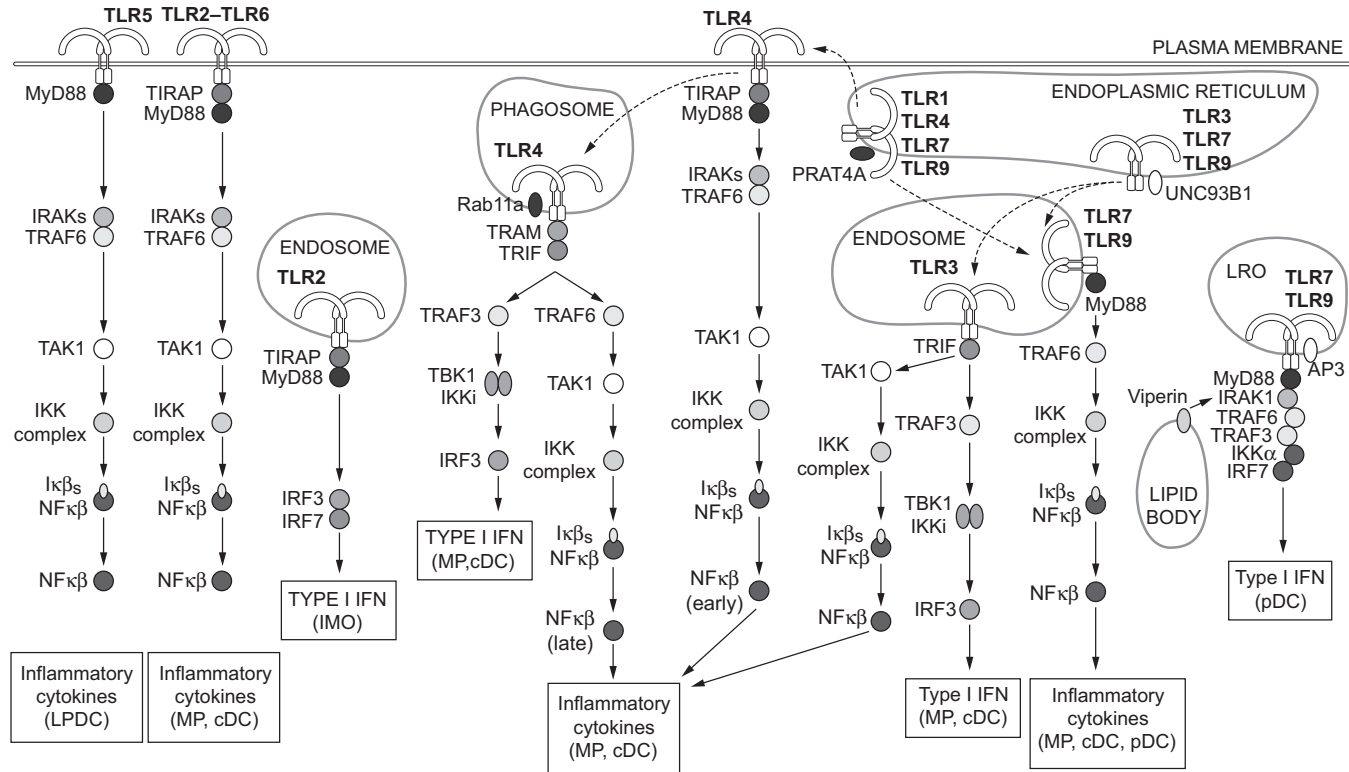


Fig. 1.1 TLR trafficking and signaling. Reprinted from *Immunity*, 34, T. Kawai and S. Akira, Toll-like receptors and their cross-talk with other innate receptors in infection and immunity, 637–650, Copyright (2011) with permission from Elsevier.

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Table 1.1 Pattern-associated molecular patterns (PAMPs) and their recognition by toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs)

Origin of PAMP	PAMP	TLR involved in recognition	Other PRRs involved in recognition
Bacteria, mycobacteria	Lipopolysaccharide	TLR4	
	Lipoproteins, lipoteichoic acid, peptidoglycan	TLR2/1, TLR2/6	NOD1-2, NALP3, NALP1
	Flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-1, MDA5, NALP3
	Structural proteins	TLR2, TLR4	
Fungi	Zymosan, β -glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	Trypanosome glycosylphosphatidylinositol-anchored mucin-like glycoproteins (tGPI-mucin)	TLR2	
	Trypanosome glycoinositolphospholipids	TLR4	
	DNA	TLR9	
	<i>Plasmodium</i> hemozoin	TLR9	NALP3
	<i>Toxoplasma gondii</i> profilin-like molecule	TLR11	

AIM, absent in melanoma; DAI, DNA-dependent activator of interferon; IFI, interferon γ -inducible protein; IPAF, interleukin-1 β -converting enzyme (ICE) protease-activating factor; MDA, melanoma differentiation-associated gene; NAIP, neuronal apoptosis inhibitory protein; NALP, NACHT-, LRR- and PYD-containing proteins; NOD, nucleotide-binding oligomerization domain; RIG, retinoic acid-inducible gene.

Source: Modified from *Immunity*, 34, T. Kawai and S. Akira, Toll-like receptors and their cross-talk with other innate receptors in infection and immunity, 637–650, Copyright (2011) with permission from Elsevier.

patterns (PAMPs). Since these molecular patterns exist in benevolent, neutral and malevolent organisms alike, they should actually be designated as MAMPs (microbial-associated molecular patterns) (Mackey and McFall, 2006). Upon MAMP/PAMP recognition, PRRs initiate a series of signaling programs that execute the first line of host defensive responses necessary for killing infectious microbes. In addition, PRR signaling simultaneously induces maturation of dendritic cells (DCs), which is responsible for alerting induction of the second line of host defense, that is, adaptive immunity.

Toll-like receptors (TLRs) were the first PRRs to be identified (Akira *et al.*, 2001). TLRs are type I transmembrane proteins with ectodomains containing leucine-rich repeats that mediate the recognition of PAMPs, transmembrane domains and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains required for downstream signal transduction. They are expressed either on the cell surface or associated with intracellular vesicles (Fig. 1.1). To date, 10 functional TLRs have been identified in humans (reviewed in Kawai and Akira, 2010). Each TLR detects distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites (Table 1.1). These include lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan, lipoteichoic acid, bacterial flagellar proteins, viral double stranded RNA and unmethylated DNA with CpG motifs characteristic of microbial DNA. Interaction between PAMPs and TLRs initiates a signaling cascade upon recruitment of an adapter protein (e.g. MyD88) leading to activation of members of the mitogen activated protein (MAP) kinase family, nuclear factor kappa B (NF κ B) and interferon (IFN) regulatory factors amongst others (Kawai and Akira, 2010).

After discovery of TLRs, several classes of cytosolic PRRs, including RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs), were identified. These PRRs, in concert with TLRs, have a critical role in both innate and adaptive immunity. A more detailed discussion of these and other ligands can be found elsewhere (Kawai and Akira, 2011).

1.3.2 Cellular components of the (innate) immune response

All cells of the immune system derive from the bone marrow; in fact, all of the inflammatory and ancillary cells that work in concert with the primary cells of the immune system derive from the pluripotent stem cell (Fig. 1.2). This pluripotential stem cell gives rise to a lymphoid stem cell and a myeloid stem cell. The lymphoid stem cell differentiates further into the four major populations of mature lymphocytes: T cells, the B cells, natural killer (NK) cells and NK-T cells. Myeloid stem cells (also termed common myeloid progenitors) give rise to several different forms of granulocytes, to megakaryocytes and platelets and to erythrocytes. Cells of the granulocyte lineage that play prominent immune roles include neutrophils, monocytes, macrophages, basophils, eosinophils and mast cells. Differentiation of the committed stem cells is critically dependent on an array of cytokine and cell–cell interactions.

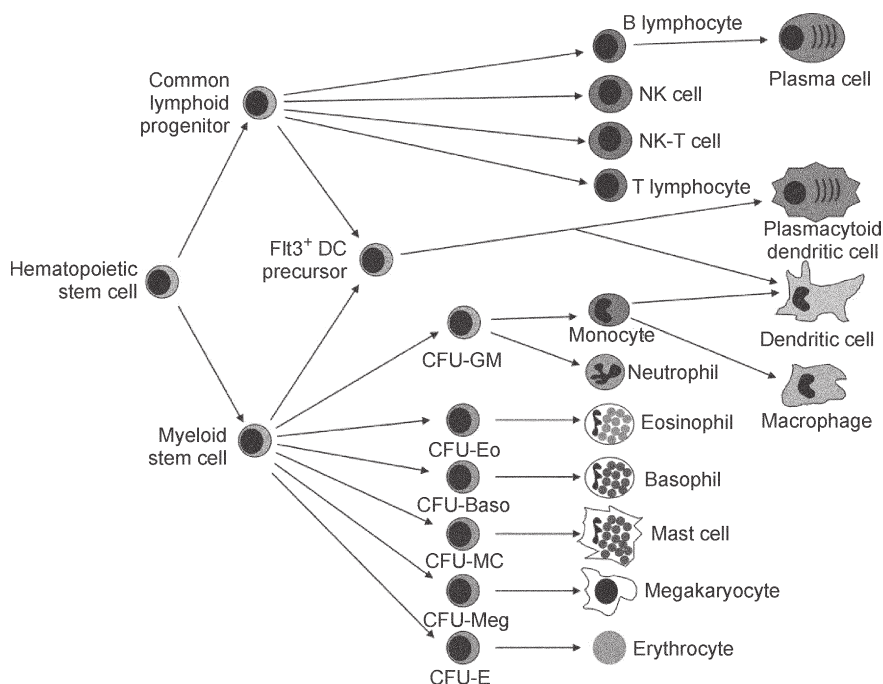


Fig. 1.2 Hematopoietic stem cell-derived lineages. Reprinted from *Journal of Allergy and Clinical Immunology*, 125, D.D. Chaplin, Overview of the immune response, S3–S23, Copyright (2010) with permission from Elsevier.

Neutrophils

A key feature of the innate response is recruitment and activation of neutrophils at the site of infection and tissue injury to eradicate bacterial pathogens. Such processes, when dysfunctional, lead to inflammatory disorders of the connective tissues, vasculitic diseases and systemic inflammatory response syndrome (Witko-Sarsat *et al.*, 2000). Neutrophils are mobile cells and flow freely in the blood as the circulating pool or roll alongside the vascular endothelium as the marginating pool. Neutrophils home to a site of infection via many steps involving pro-inflammatory mediators, adhesion molecules, chemoattractants, and chemokines (see below). The recruited neutrophils phagocytose organisms by extending pseudopodia (projections of cytoplasmic membranes) which form a membrane-bound vesicle (phagosome) around the particle. This fuses with neutrophil cytoplasmic granules to form the phagolysosome. In this enclosed compartment killing occurs via two mechanisms:

1. **The oxygen-dependent response or respiratory burst:** this mechanism involves the reduction of oxygen by an NADPH oxidase leading to the production of toxic oxygen metabolites such as singlet oxygen, hydrogen peroxide and hydroxyl radicals (von Andrian and Mackay, 2000).
2. **The oxygen-independent response:** this method uses the highly toxic cationic proteins and enzymes such as myeloperoxidase and lysozyme, which are contained in the cytoplasmic granules.

The ingestion and killing of an organism is more effective if it is opsonized with specific antibody or complement (C). These molecules bind to neutrophil Fc and C receptors, increasing adhesion between particle and phagocyte and priming the cell for activation (Garred *et al.*, 1995). Some encapsulated organisms, such as pneumococcus and haemophilus, are not susceptible to neutrophil phagocytosis unless first coated with antibody. This is why individuals with antibody deficiency are susceptible to this type of infection, despite normal neutrophil numbers and function.

Monocytes and macrophages

Macrophages are derived from the monocyte lineage and contain receptors for carbohydrates which are able to distinguish between 'foreign' and 'self' molecules. Like neutrophils, macrophages contain receptors for antibodies and complement and, as such, micro-organisms coated with antibodies and/or complement enhance phagocytosis. Once ingested, the micro-organisms are attacked by toxic intracellular molecules such as anions, radicals, nitric oxide, cationic peptides and proteins and lysozymes as described for neutrophils. Macrophages also produce large amounts of cytokines, such as IL-12 and IFN- γ , giving them a regulatory role in adaptive immune responses. The highly phagocytic monocytes and macrophages remove dead and dying cells in necrotic tissue which release substances that trigger an inflammatory response. Cells which are dying by virtue of apoptosis (programmed cell death resulting in the digestion of DNA by endonucleases) express molecules on their cell surface which promote them as candidates for phagocytosis.

Eosinophils

Eosinophils, the prominent leukocytes in allergic inflammation and antihelminthic responses, are characterized by an abundance of intracellular granules that contain preformed proteins including distinct cationic proteins, such as eosinophil cationic protein (ECP), and a wide range of preformed cytokines, chemokines and growth factors. Eosinophils are weakly phagocytic and, when activated, kill parasites by releasing cationic proteins and reactive oxygen metabolites into the extracellular fluid. They secrete leukotrienes, prostaglandins and various cytokines. Cell-free eosinophil granules are found extracellularly as intact membrane-bound organelles in sites associated with infiltration, but their functional capacities are unknown. Neves *et al.* (2010) reported that receptors for cysteinyl leukotrienes (cysLTs), cysLT1R, cysLT2 receptor and the purinergic P2Y12 receptor, are expressed on eosinophil granule membranes. Leukotriene (LT) C₄ and extracellularly generated LTD₄ and LTE₄ stimulated isolated eosinophil granules to secrete eosinophil cationic protein. Inhibitors of the P2Y12 receptor and cysLT1R inhibited these effects. These studies have identified previously unrecognized sites of localization, the membranes of intracellular eosinophil granule organelles and the function for cysLT-responsive receptors that mediate cysLT-stimulated secretion from within eosinophil granules.

Basophils and mast cells

Basophils and mast cells are morphologically similar cells that represent distinct lineages. By virtue of the cell-surface expression of high-affinity receptors for IgE (FcεRI), they are key initiators of immediate hypersensitivity responses and the host response to helminthic parasites, releasing histamine and other preformed mediators from their granules and producing important quantities of lipid mediators that stimulate tissue inflammation, edema and smooth muscle contraction. Importantly, basophils (and, to a lesser extent, mast cells) can release substantial amounts of systemic IL-4 and IL-13. It has been suggested that these cells can act as APCs for initiating Th2 responses (Sokol *et al.*, 2009) whilst others have suggested an amplifying role for pre-existing antigen-specific Th2 responses (Hammad *et al.*, 2010).

Natural killer cells

NK cells are defined morphologically as large granular lymphocytes. They are distinguished by their lack of either T-cell receptor (TCR) or surface immunoglobulin. NK cells have the ability to destroy infected and malignant cells using a complex collection of activating and inhibitory cell surface receptors (Jonsson and Yokoyama, 2009). NK cells may bind the Fc receptors that bind IgG (FcγR) and initiate antibody-dependent cellular cytotoxicity of the target cell. Alternatively, killer-activating receptors and killer-inhibitory receptors of NK cells may be used to bind target cells and shape the subsequent NK cell response. The killer-activating receptors recognize a number of different molecules present on the surface of all nucleated cells, whereas the killer-inhibitory receptors recognize MHC class I molecules on all nucleated cells. If the killer-activating signal is released then the NK cells destroy the target cell unless overridden by the killer-inhibitory signal upon recognition of the target cell MHC class I molecules. The lack of the MHC class I receptor expression (due to infection or malignancy) ensures there is no inhibitory signal via the killer-inhibitory receptor and thus the NK cell will eliminate the cell with the abnormal phenotype. This is accomplished by releasing and then inserting the pore-forming molecule, perforin, into the membrane of the target cell and releasing cytotoxic granzymes through it.

1.3.3 Soluble factors in innate defense

The innate immune response involves the release of products such as complement, acute-phase proteins, cytokines, IFNs, reactive molecules (respiratory burst) and pro-inflammatory mediators such as leukotrienes and histamine. Other soluble factors, such as immunoglobulins, surfactant proteins (Madsen *et al.*, 2000), Clara cell proteins (Braido *et al.*, 2007) and palate, lung and nasal epithelium clones (PLUNCs) (Bingle *et al.*, 2005), also contribute to innate defense. Together they are involved in the important pro-inflammatory response of phagocytosis and degranulation.

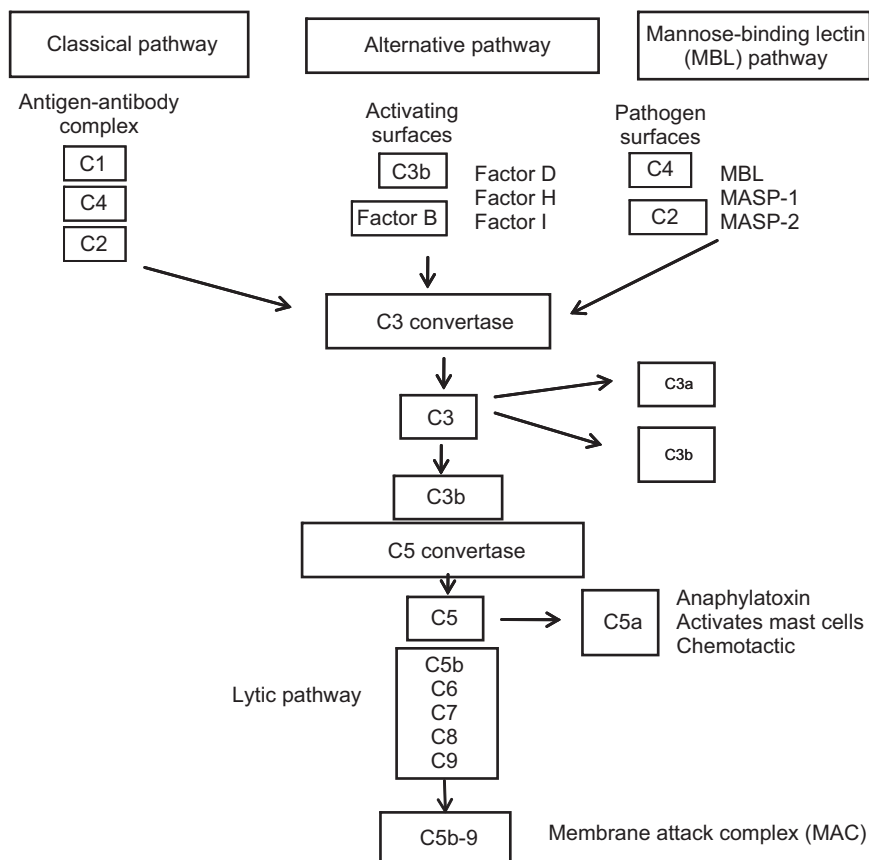


Fig. 1.3 The activation pathways of complement.

Complement

The early events of the complement cascade can be activated in three ways: (i) The classic pathway by antigen–antibody complexes; (ii) The alternative pathway by microbial cell walls; and (iii) The lectin pathway by the interaction of microbial carbohydrates with mannose-binding protein in the plasma (Fig. 1.3). Once initiated many immunologically active substances are released. Complement component C3, via its proteolytic-cleavage fragment C3b molecule, is rested on the surface of micro-organisms and phagocytosis of the microbe is initiated. The remaining complement fragments, C3a, C4a and C5a, instigate the release of inflammatory mediators from mast cells. C5a will also attract neutrophils to inflamed tissues. The complement components C5b, C6, C7, C8 and C9 finally form the membrane-attack complex, which perforates target cell membranes and thereby promotes death of the target cell.

Acute-phase proteins

The acute-phase proteins work together to resist infection and promote damage repair. Plasma levels of these proteins change in response to infection, inflam-

mation and damaged tissue. Examples of acute-phase proteins include C-reactive protein, serum amyloid A protein, proteinase inhibitors and coagulation proteins.

1.3.4 Adhesion molecules and signaling

In order for immune cells to work effectively, they need to be recruited to sites of inflammation and appropriately activated. Cellular recruitment and activation is facilitated through the complex interplay between cell adhesion and signaling (cytokines and chemokines) molecules.

Adhesion molecules are surface-bound molecules involved in cell-to-cell interactions. Their main function is in facilitating processes where close contact of cells is required (e.g. cell migration, phagocytosis and cellular cytotoxicity). Adhesion molecules associate with cytoplasmic proteins and cytoskeletal components to cause cytoskeletal reorganization, allowing cells to undergo direct movement between other cells. Ligation of the adhesion molecule leads to signal transduction, cell activation, alternation in receptor expression and cytokine production, and it affects cell survival. Cells can express adhesion molecules independently or upregulate them on exposure to cytokines, chemokines or other pro-inflammatory molecules such as complement activation products and microbial metabolites. Adhesion molecules are expressed on both leucocytes and endothelial cells, enabling interaction between the two (Fig. 1.4).

There are several families of adhesion molecules based on structure and function (Parkin and Cohen, 2001) (Table 1.2). The main ones are the intercellular

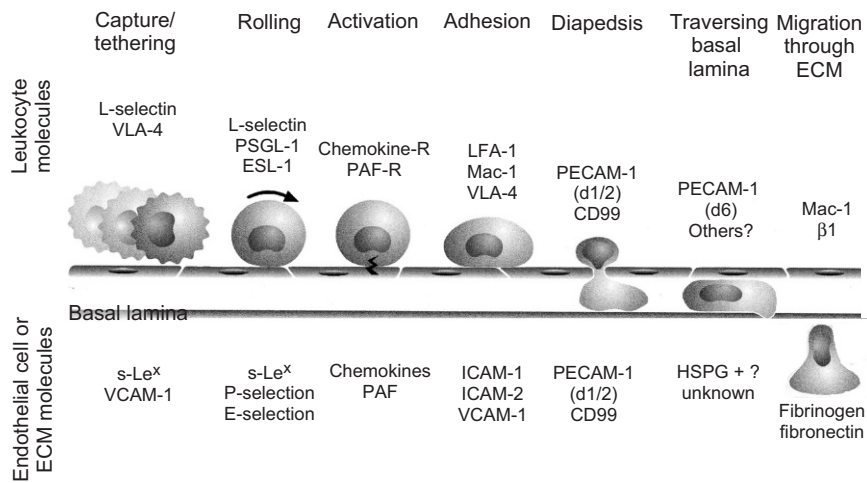


Fig. 1.4 Leukocyte–endothelial cell interactions. Reprinted with permission from Macmillan Publishers Ltd: *Laboratory Investigation* (W.A. Muller, Leukocyte–endothelial cell interactions in the inflammatory response, 82, 521–533), copyright 2002. ECM, extracellular matrix.

Table 1.2 Summary of the structure and ligands of the major cell adhesion molecules

Cell adhesion molecule	Common name	Ligand
<i>Selectin family</i>		
CD62L	L-selectin	CD34, MAdCAM-1, GlyCAM-1, s-Le ^x , Sgp200
CD62E	E-selectin	CD65, CD66, L-selectin
CD62P	P-selectin	PSGL-1 (CD162)
<i>Integrin family</i>		
CD (Subunit)		
CD49a/29 ($\alpha_1\beta_1$)	VLA-1	Laminin, collagen
CD49b/29 ($\alpha_2\beta_1$)	VLA-2	Laminin, collagen
CD49c/29 ($\alpha_3\beta_1$)	VLA-3	Laminin, collagen, fibronectin
CD49d/29 ($\alpha_4\beta_1$)	VLA-4	VCAM-1, fibronectin CS-1 domain
CD49e/29 ($\alpha_5\beta_1$)	VLA-5	Fibronectin
CD49f/29 ($\alpha_6\beta_1$)	VLA-6	Laminin
CD11a/18 ($\alpha_L\beta_2$)	LFA-1	ICAM-1 to -5
CD11b/18 ($\alpha_M\beta_2$)	Mac-1	ICAM-1 and -2, C3bi, fibrinogen, heparin
CD11c/18 ($\alpha_X\beta_2$)	p150, 95	C3bi, fibrinogen
CD α_d /18 ($\alpha_d\beta_2$)		ICAM-3, VCAM-1
CD51/61 ($\alpha_v\beta_3$)	Vitronectin receptor	Vitronectin, PECAM-1, other RGD peptides
CD49d/ β_7 ($\alpha_4\beta_7$)	LPAM-1	MAdCAM-1, VCAM-1, fibronectin CS-1 domain
CD103/ β_7 ($\alpha_E\beta_7$)	HML-1	E-cadherin
<i>Immunoglobulin superfamily</i>		
CD54	ICAM-1	LFA-1, fibrinogen, Mac-1
CD102	ICAM-2	LFA-1
CD50	ICAM-3	LFA-1, $\alpha_d\beta_2$
CD106	VCAM-1	VLA-4, $\alpha_4\beta_2$
CD31	PECAM-1	$\alpha_v\beta_3$, HSPG

CD, cluster of differentiation nomenclature; C3, complement factor 3; CS-1, connecting segment-1; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; HML, human mucosal lymphocyte antigen-1; HSPG, heparin sulfate proteoglycan; ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; LPAM-1, lymphocyte peyer's patch adhesion molecule-1; Mac-1, macrophage-1 antigen; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PECAM, platelet-endothelial cell adhesion molecule; PSGL-1, P-selectin glycoprotein ligand-1; RGD, arginine-glycine-aspartic acid; s-Le^x, sialyl-Lewis^x carbohydrate antigen; Sgp200, sulfated glycoprotein 200; VCAM, vascular cell adhesion molecule, VLA, very late (activation) antigen.

adhesion molecules, integrins, selectins and cadherins (calcium-dependent adherins). As well as molecules on leucocytes and vascular endothelium, there are also tissue-specific adhesion molecules called addressins. These target lymphocytes to particular groups of lymphoid tissue such as in the gut, lung, skin, peripheral lymph nodes, brain and synovium.

Chemokines

Chemokines are particular members of the cytokine family that play a key role in leucocyte migration (Table 1.3) (Parkin and Cohen, 2001). They have marked chemotactic function and are named by the position of the two cysteine (C)

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14 Diet, immunity and inflammation

Table 1.3 Chemokine and chemokine receptor superfamily

Chemokine	Common name(s)	Receptor(s)
<i>CC chemokines</i>		
CCL1	I-309	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1 α	CCR1, 5
CCL4	MIP-1 β	CCR5
CCL5	RANTES	CCR1, 3, 5
CCL6	–	Unknown
CCL7	MCP-3	CCR1, 2, 3
CCL8	MCP-2	CCR3
CCL9/10	–	Unknown
CCL11	Eotaxin	CCR3
CCL12	–	CCR3
CCL13	MCP-4	CCR2, 4
CCL14	HCC-1	CCR1
CCL15	HCC-2/MIP-1 δ	CCR1, 3
CCL16	HCC-4/LEC	CCR1
CCL17	TARC	CCR4
CCL18	DC-CK1/PARC	Unknown
CCL19	MIP-3 β /ELC	CCR7
CCL20	MIP-3 α /LARC	CCR6
CCL21	6Ckine/SLC	CCR7
CCL22	MDC/STCP-1	CCR4
CCL23	MPIF-1	CCR1
CCL24	Eotaxin-2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3
CCL27	CTAP/ILC	CCR10
<i>CXC chemokines</i>		
CXCL1	GRO- α /MGSA- α	CXCR2 > CXCR1
CXCL2	GRO- β /MGSA- β	CXCR2
CXCL3	GRO- γ /MGSA- γ	CXCR2
CXCL4	PF-4	Unknown
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3
CXCL10	IP-10	CXCR3
CXCL11	I-TAC	CXCR3
CXCL12	SDF-1	CXCR4
CXCL13	BLC/BCA-1	CXCR5
CXCL14	BRAX/Bolekine	Unknown
CXCL15	–	Unknown
CXCL16	CXCL16	CXCR6
<i>C chemokine</i>		
XCL1	Lymphotactin, SCM-1 α	XCR1
<i>CX₃C chemokine</i>		
CX ₃ CL1	Fractalkine, neurotatin	CX ₃ CR1

(Continued)

Table 1.3 Continued

BCA-1, B cell-activating chemokine-1; BLC, B lymphocyte chemoattractant; BRAK, breast and kidney chemokine; C, N-terminal cysteine (C) residue; CTAP, connective tissue-activating peptide; DC-CK, dendritic cell-derived chemokine-1; ELC, EB11 ligand chemokine; ENA, epidermal cell-derived neutrophil chemotactic activity; GCP, granulocyte chemotactic protein; GRO, growth factor-related oncogene; HCC, hemofiltrate-derived CC chemokine; I-TAC, interferon-inducible T cell α chemoattractant; IL-8, interleukin-8; ILC, IL-11 R- α -locus chemokine; IP-10, inducible protein-10; LARC, liver activation regulated chemokine; LEC, liver-expressed chemokine; MCP, monocyte chemotactic protein (peptide); MDC, macrophage-derived chemokine; MGSA, melanocyte growth-stimulating activity; Mig, monokine induced with IFN(γ); MIP, macrophage inflammatory protein; MPIF, myeloid progenitor inhibitory factor-1; MRP, MIP-related protein; NAP-2, neutrophil-activating peptide; PARC, pulmonary and activation-regulated chemokine; PF-4, platelet factor 4; RANTES, regulated on activation, normal T cells expressed and secreted; SLC, secondary lymphoid-tissue chemokine; STCP-1, stimulated T cell chemoattractant protein-1; SCM-1 α , single C motif-1/ α ; SDF-1, stromal cell-derived factor-1; TARC, thymus- and activation-regulated chemokine; TECK, thymus-expressed chemokine; X, any amino acid.

residues compared with the other amino acids (X). The two main subgroups are CXC (α -chemokines) and CC (β -chemokines). Chemokines are produced by most cells on stimulation with pro-inflammatory cytokines or bacterial products, and chemokine receptors are found on all leucocytes. The effects of chemokines are more prolonged than other chemoattractants such as complement activation products, as they bind to glycosaminoglycans on cell surfaces and the extracellular matrix. Chemokines that cause recruitment of leucocytes are termed inflammatory. Additionally, there are lymphoid chemokines which regulate leucocyte positioning within the spleen and other lymphoid tissues.

Cytokines

Cytokines are small molecular weight proteins which act as soluble mediators and as 'messengers' secreted by one cell to alter its own behavior or that of another cell. As such, they are regulators of immune responses, working within the immune system and between the immune system and other complex cellular networks within other tissues in the body (Table 1.4) (Parkin and Cohen, 2001). Cytokines send intracellular signals by binding to specific cell surface receptors. Although most are the soluble form of a cytokine receptor, some may be membrane-anchored, making the differentiation between cytokine and receptor roles very difficult to define. Cytokines are produced by virtually all cells and have a wide variety of functions. The biological effect depends on the cytokine and the cell involved, but normally cytokines affect cell activation, division, apoptosis or movement. They act as autocrine, paracrine or endocrine messengers. Cytokines produced by leucocytes and which have effects mainly on other white cells are termed *interleukins*. Cytokines that have a chemoattractant activity are called *chemokines*. Those that cause differentiation and proliferation of stem-cells are called *colony-stimulating factors*. Some cytokines also have a role in defense and interfere with viral replication and, as such, are called *interferons*. IFNs released by virally infected cells create a barrier around neighbouring cells and create viral resistance. Cytokines and their antagonists are being used increasingly for

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Table 1.4 Summary of the main sources and effects of cytokines

Cytokine	Main source(s)	Primary effects
<i>IL-1 family</i>		
IL-1 α	Macrophages, epithelial cells	Cell proliferation and differentiation
IL-1 β	Monocytes/macrophages	Cell proliferation, differentiation and apoptosis; induction of COX-2
IL-1RA	Monocytes/macrophages	Competitively inhibits the binding of IL-1 to cell surface receptors
IL-18	Macrophages, Kupffer cells	Similar to IL-12, inhibits IgE production by increasing IFN- γ
IL-33	Fibroblasts, epithelial cells, endothelial cells	Induction of Th2 cytokines such as IL-4, IL-5 and IL-13 by Th2 cells, basophils and mast cells
<i>IL-10 family</i>		
IL-10	Monocytes, macrophages, T cell subsets, DCs, B and NK cells, mast cells, neutrophils, eosinophils	Differentiation to macrophages; inhibits expression of MHC class II and many adhesion molecules; inhibits IFN- γ and TNF production resulting in switch of T cell differentiation from Th1 to Th2
IL-19	Monocytes, macrophages, tissue cells (e.g. keratinocytes)	Enhanced expression in IL-4 (or IL-13), but not IFN- γ
IL-20	Monocytes, mature DCs, tissue cells	?
IL-22	T and NK cell subsets	Induction of acute-phase reactants
IL-24	Monocytes, T cells, tissue cells	?
IL-26	T and NK cell subsets	?
<i>IL-12 family</i>		
IL-12	DCs, monocytes, macrophages, B cells	Th1 activation/maintenance; blocks Th2
IL-23	DCs, monocytes, macrophages, B cells	Th1 activation; Th17 polarization/proliferation
IL-27	DCs, monocytes, macrophages, B cells	Th1 cell proliferation, skewing effector T cell lineages; blocks Th17
IL-35	FoxP3 ⁺ Tregs, others?	Inhibits T cell proliferation; additional?

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IL-17 family

IL-17	CD4 ⁺ cells (memory)	Autocrine proliferation and activation; secretion of IL-6, IL-8, G-CSF, GRO α , PGE2; expression of ICAM-1
IL-17B	?	Release of TNF- α and IL-1 β
IL-17C	?	Release of TNF- α and IL-1 β
IL-17D	CD4 ⁺ cells	Expression of IL-6, IL-8, CSF2 and GM-CSF
IL-17E (IL-25)	CD4 ⁺ cells	Induction of Th2 cytokines, IL-4 and IL-13
IL-17F	CD4 ⁺ cells	Secretion of IL-6, IL-8, G-CSF, GRO α , PGE2 and TGF β

IFN family

IFN- α	Monocytes, macrophages, pDC	Inhibits viral replication
IFN- β	Monocytes, macrophages	Similar to IFN- α
IFN- γ	CD4 ⁺ Th1, lymphocytes, NK cells and some CD8 ⁺ lymphocytes	Differentiation; activation to express Fc γ R, MHC I and II, nitric oxide synthase, IL-1 and TNF

TNF family

TNF- α	Monocytes/macrophages	Enhanced apoptosis; enhanced cytokine, MHC I and II, adhesion molecule expression; cytotoxicity
TNF- β	T cells	Immune activation and generalized vascular effects (also known as lymphotoxin)

Other cytokines

IL-2	CD4 ⁺ cells	Clonal expansion of Ag-specific cells; differentiation and cytokine expression; maturation of CD8 ⁺ cells
IL-3	T cells	Proliferation of differentiation
IL-4	CD4 ⁺ and CD8 ⁺ T cells	Growth and activation; production of MHC II, IL-6, TNF, CD23, CD72; switch factor for IGE and IgG1
IL-5	CD4 ⁺ and CD8 ⁺ lymphocytes	Proliferation, chemoattraction, adhesion, activation, enhanced survival and degranulation
IL-6	Monocytes/macrophages	Differentiation into plasma cells; switch factor for IgG1 and IgA; induces acute-phase protein synthesis
IL-7	Bone marrow stromal cells	Proliferation
IL-8	Macrophages	Directs migration (i.e. chemoattractant) but inhibits adhesion to endothelium

(Continued)

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Table 1.4 Continued

Cytokine	Main source(s)	Primary effects
IL-9	CD4 ⁺ T cells	Enhanced response to IL-4; stimulates T cell proliferation; enhances IgE production
IL-11	Bone marrow stromal cells	Similar to IL-6
IL-13	CD4 ⁺ Th2 lymphocytes	Similar to IL-4, enhances MHC II and integrins
IL-14	Activated T lymphocytes	Proliferation; suppresses Ig secretion
IL-15	Monocytes/macrophages	Proliferation; increased cytotoxicity and ICAM-3
IL-16	CD8 ⁺ lymphocytes	Chemoattractant; growth factor
IL-21	Activated CD4 ⁺ T cells	Induction of CD40-dependent B cell proliferation; inhibition of NK cell response; proliferation of naïve T cells and bone marrow progenitor cells (requires IL-15)
G-CSF	Monocytes, fibroblasts, epithelial cells	Maturation and differentiation
M-CSF	Monocytes, fibroblasts, epithelial	Monocyte differentiation
PDGF	Platelets; monocytes/macrophages	Proliferation; chemoattractant for fibroblasts; active in wound healing, atherogenesis and airway remodeling
SCF (<i>c-kit</i> ligand; mast cells growth factor)	Bone marrow stroma; fibroblasts	Chemoattractant; with IL-3 stimulates growth; has histamine-releasing activity
GM-CSF	Activated macrophages; T cells	Proliferation, differentiation, survival and enhanced cytokine production
TGF-β	Platelets	Inhibits IL-2-stimulated growth (T cells); switch factor for IgA but inhibits IgM and IgG; counteracts IL-4 stimulation of IgE (B cells); inhibits cytotoxicity (NK cells)

COX, cyclooxygenase; CSF, colony stimulating factor; DC, dendritic cell; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; GRO, growth factor-related oncogene; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; M-CSF, monocyte colony stimulating factor; MHC, major histocompatibility complex; NK, natural killer; PDGF, platelet-derived growth factor; PGE, prostaglandin E; SCF, stem cell factor; TGF, transforming growth factor-β; TNF, tumor necrosis factor.

therapeutic agents such as interleukin (IL)-2 and IFN- α for the treatment of melanoma (Nicholas and Lesinski, 2011).

1.4 Adaptive immunity

1.4.1 Introduction

Adaptive immunity against pathogens originates with vigorous clonal expansion of antigen-specific T lymphocytes that recognize their cognate antigens on the surface of DCs in secondary lymphoid organs. Once primed, effector T cells (e.g. CD4⁺ T cells) migrate to follicles to help B cells produce antibodies, and to peripheral sites of antigen exposure to fight incoming pathogens by ensuring the appropriate type of effector cell function. Type 1 effector (Th1) cells produce IFN- γ that promotes clearance of viruses and intracellular bacteria, while Th2 cells produce IL-4, IL-5 and IL-13 that promote clearance of extracellular parasites. Once antigen is eliminated, central memory (T_{CM}) and effector memory (T_{EM}) T cells persist in the memory pool to provide systemic immune surveillance in lymphoid organs and in peripheral non-lymphoid tissues to react promptly in case of re-attack. To do this, T cells not only keep 'memory' of the cytokines to be produced, but also for the site where effector function is needed. This section summarizes current knowledge of the various components necessary for orchestrating the adaptive immune response.

1.4.2 Antigen recognition by T lymphocytes/major histocompatibility molecules

The biologic basis for antigen recognition in the context of major histocompatibility (MHC) molecules is to allow distinction between self and non-self. In humans, the MHC gene complex is located on chromosome 6 and comprises genes that code for human leukocyte antigen (HLA) (Klein and Sato, 2000). MHC molecules are cell-surface glycoproteins that bind peptide fragments of proteins that have either been synthesized within the cell (class I MHC molecules) or that have been ingested by the cells and proteolytically processed (class II MHC molecules).

Class I and II MHC molecules

Class I MHC molecules (HLA-A, HLA-B, HLA-C) are composed of a 44 kD variant chain that is non-covalently associated with the 12 kD non-MHC invariant chain, β_2 -microglobulin (Bjorkman, 1997). Class II MHC molecules (HLA-DR, HLA-DQ, HLA-DP) are composed of a 34 kD α -variant chain, non-covalently associated with a 29 kD β -variant chain (Bjorkman, 1997). The biologic role of the MHC molecules is to display antigenic peptides that enable appropriate TCRs to bind them. In general, class I MHC molecules present endogenously derived antigenic peptide after antigen processing, such as viral epitopes, to CD8⁺ T cells,

whereas class II MHC molecules present exogenously derived antigenic peptides, such as soluble bacterial protein-derived antigenic peptide, to CD4⁺ T cells.

Class I MHC molecules have three external domains, and their crystalline structure has been resolved (Bjorkman *et al.*, 2005). The antigen-binding site resides within a groove formed by the first and second (α_1 and α_2) external domains of the class I MHC molecule, and the appropriate TCR on CD8⁺ T cells recognizes the antigen in association with these epitopes; the α_3 domain has been implicated in the interaction with CD8. This restricts recognition of antigenic peptides that are presented in class I HLA molecules to CD8⁺ cytolytic T cells. On class II MHC molecules the α and β chains each have two immunoglobulin-like external domains. The crystalline structure of class II molecule has also been determined, demonstrating a putative antigen-binding cleft on the distal face of the molecule (Brown *et al.*, 1993). The appropriate TCR on CD4⁺ T cells recognizes the antigen in this binding cleft, whereas the CD4 molecule binds to a non-polymorphic epitope or epitopes on the class II MHC molecule.

Class I proteins are expressed on all nucleated cells, in contrast, class II proteins are expressed constitutively on B cells, DCs, monocytes and macrophages, all cells that present antigens to CD4⁺ T cells. Expression of MHC class II proteins can also be induced on many additional cell types, including epithelial and endothelial cells after stimulation with IFN- γ , permitting these cells to present antigens to CD4⁺ T cells at sites of inflammation.

HLA-independent presentation of antigen

Antigen presentation by class I and class II MHC molecules to CD8⁺ and CD4⁺ T lymphocytes is limited to protein antigens. It is now well accepted that there is a class of T cells that recognize bacterial lipid antigens that are presented bound to CD1 molecules (Brigl and Brenner, 2004). Human CD1 molecules bind and display or present lipid and glycolipid antigens from mycobacteria for recognition by T cells. Presentation requires uptake of antigen into endosomes, where it binds to CD1. T-cell recognition of CD1-presented non-peptide antigens is a newly defined immune response that could be important for host defense against a variety of pathogens.

1.4.3 T lymphocytes

T cell development

Developing T cells within the thymus undergo rigorous selection to ensure the usefulness and safety of mature T cells bearing rearranged $\alpha\beta$ TCRs. Thymocytes at the CD4⁺CD8⁻ double-negative stage undergo recombination activation gene (RAG)-mediated TCR β gene rearrangement, and generation of a functional TCR β -chain induces proliferation and CD4 and CD8 co-receptor expression. At the CD4⁺CD8⁺ double-positive stage, a second wave of RAG-dependent rearrangement, this time at the TCR α locus, generates rearrangements that encode TCR α -chains that pair with the already available TCR β -chains, and these TCR $\alpha\beta$

pairs are tested for interactions with self-MHC molecules. Useful affinity for self-MHC class II (MHC II) and class I molecules drives positive selection, upregulation of TCR surface expression and commitment to the CD4 and CD8 T-cell lineages, respectively. Insufficient interactions with MHC result in death by neglect. Positively selected CD4 single-positive and CD8 single-positive thymocytes test their TCRs against a broad array of self-MHC–self-peptide ligands for self-reactivity, and those cells that recognize self are removed from the repertoire by apoptosis (reviewed in Starr *et al.*, 2003). After thymic exit, basal TCR signaling in the lymphoid periphery promotes survival and homeostatic proliferation and is an additional selective force that maintains useful components of the peripheral T-cell repertoire (reviewed in Seddon and Zamoyska, 2003).

Approximately 90–95% of circulating T cells use the $\alpha\beta$ TCR described above. The other 5–10% use an alternate heterodimeric TCR composed of γ and δ chains. The γ and δ chains also assemble by means of RAG1/RAG2-mediated rearrangement of V, D (for the δ chain only) and J elements. A portion of the $\gamma\delta$ T cells is generated in the thymus, but a major fraction appears to be generated in an extrathymic compartment, resulting in cells that largely populate the gastrointestinal tract (Ishikawa *et al.*, 2007).

T cell-antigen receptor complex

The antigen-specific α and β chains of the TCR associate with invariant accessory chains that serve to transduce signals when the TCR binds to antigen-MHC complexes. These accessory chains make up the CD3 complex, consisting of the transmembrane CD3 γ , CD3 δ and CD3 ϵ chains plus a largely intracytoplasmic homodimer of 2 CD3 ζ chains. Although the stoichiometry of the CD3 complex is not definitively established, it appears that each TCR $\alpha\beta$ pair associates with a CD3 $\gamma\epsilon$ heterodimer, a CD3 $\delta\epsilon$ heterodimer and a CD3 ζ homodimer.

Interaction of the TCR/CD3 complex with antigenic peptide presented in an HLA molecule provides only a partial signal for cell activation (signal 1). Full activation requires the additional participation of a co-stimulatory molecule (signal 2), such as CD28 on the T cell and CD80 (also designated B7.1) or CD86 (B7.2) on the APC and cytokine/receptor interaction (signal 3). In fact, interaction of peptide-MHC with the TCR without a co-stimulator can lead to an anergic state of prolonged T cell non-responsiveness.

The cytoplasmic portions of each of the CD3 chains contain sequence motifs designated immunoreceptor tyrosine-based activation motifs (ITAM). When key tyrosines in these ITAMs are phosphorylated by the receptor-associated kinases Lck and Fyn, this initiates an activation cascade involving the proteins ζ chain-associated protein kinase 70 (ZAP-70), and, farther downstream, linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76 kDa (SLP-76). Activation of these proteins leads to stimulation of phospholipase C (PLC), activation of the G proteins Ras and Rac, and both protein kinase C (PKC) and the MAP kinases. Together, this complex of activation events leads to activation of genes that control lymphocyte proliferation and differentiation.

In the three-signal model of T-cell signaling (above), the outcome of antigen

recognition is determined by the integration of multiple cues in the immune microenvironment. Recently, an increasingly important role for the mammalian target of rapamycin (mTOR) in directing T-cell activation and differentiation has become apparent (reviewed in Powell and Delgoffe, 2010). This review specifically highlights interesting observations demonstrating the link between the unique metabolic requirements of T cells (including γ - δ , and α - β T cell), the ability of mTOR to integrate environmental cues to direct differentiation, and function. In addition, mTOR has been implicated in neutrophil, monocyte, DC and B cell function (Delgoffe and Powell, 2009).

T cell subpopulations

Both CD4⁺ and CD8⁺ T cells differentiate into functionally distinct subsets after exposure to antigen (Fig. 1.5). This is best described for the transition of CD4⁺ T cells from naïve to effector populations. Upon activation by TCR and cytokine-mediated signaling, naïve CD4 T cells may differentiate into at least four major types of T helper (Th) cells, Th1, Th2, Th17 and inducible T regulatory (iTreg) cells, which play a critical role in orchestrating adaptive immune responses to various micro-organisms. They can be distinguished by their unique cytokine production profiles and their functions. IFN- γ -secreting Th1 cells are effective at combating intracellular bacteria and viruses, by means of the activation of macrophages and NK cells and expansion of cytotoxic T cells (CTLs). In contrast, Th2 cells, whose cytokines (IL-4, IL-5 and IL-13) direct immunoglobulin E (IgE)- and eosinophil-mediated destruction of pathogens, are effective at controlling helminths. Although helminths are considered the prototypic Th2-inducing stimuli, bacteria, viruses and allergens can also induce Th2 responses. In addition, Th17 cells are responsible for controlling extracellular bacteria and fungi through their production of IL-17a, IL-17f and IL-22, while iTregs, together with naturally-occurring T regulatory (nTreg) cells, are important in maintaining immune tolerance (see below), as well as in regulating lymphocyte homeostasis, activation and function.

The major determinant for Th cell differentiation is the cytokine milieu at the time of antigen encounter, although the nature of cognate antigen and its affinity to the TCR as well as the available co-stimulants, many of which regulate initial cytokine production, can influence Th cell fate. IL-12 and IFN- γ are two important cytokines for Th1 differentiation. For Th2 differentiation, many cytokines, including IL-4, IL-2, IL-7 and thymic stromal lymphopoietin (TSLP), may be involved. While transforming growth factor (TGF)- β induces Th17 differentiation in the presence of IL-6, it also promotes iTreg cell differentiation when IL-2 but not IL-6 is available. In general, more than one cytokine is required for differentiation to any particular phenotype and cytokines that promote differentiation to one lineage may suppress adoption of other lineage fates.

Transcription factors are critical for Th cell differentiation and cytokine production. Cell fate determination in each lineage requires at least two types of transcription factors, the master regulators and the signal transducer and activator of transcription (STAT) proteins. The activity of the master regulators is controlled

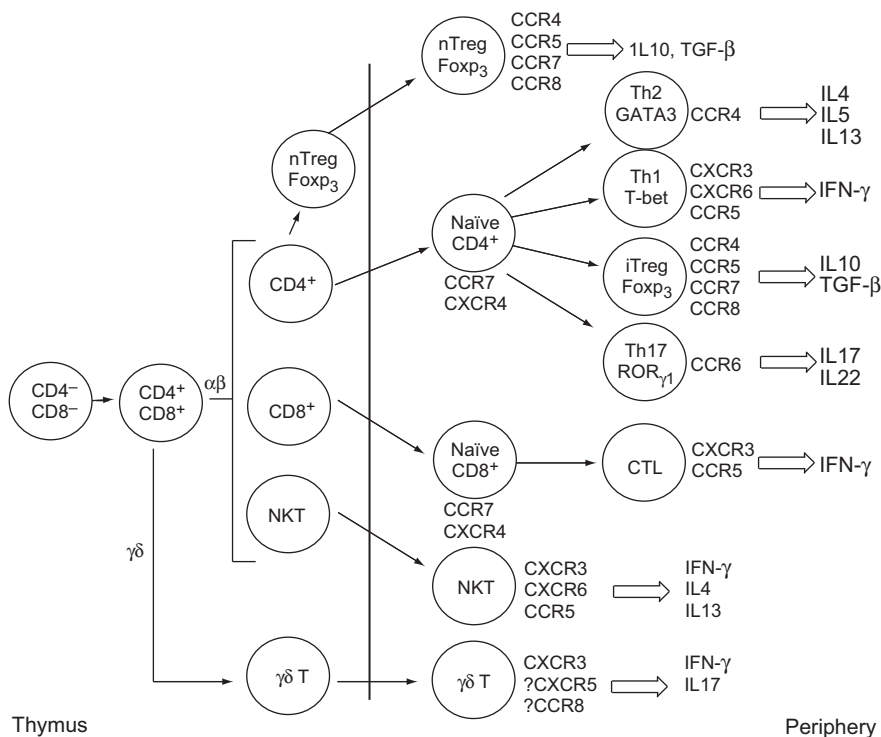


Fig. 1.5 Development of T cell subsets. Reprinted from R. Afshar, B.D. Medoff and A.D. Luster (2008) Allergic asthma: a tale of many T cells. *Clinical and Experimental Allergy* 38, 1847–1857 with permission from John Wiley & Sons.

by their expression, whereas STAT proteins are activated by cytokines through post-transcriptional modifications such as phosphorylation. The essential transcription factors of Th lineages are T-bet/STAT4 (Th1), GATA-3/STAT5 (Th2), ROR γ /STAT3 (Th17) and Foxp3/STAT5 (iTreg), respectively.

Other T helper-cell subsets

Follicular helper T cells (T_{FH}) residing in B cell follicles are essential for the generation of high-affinity isotype switched antibodies and B cell memory. Although all CD4⁺ T cells migrate to follicular regions, T_{FH} cells preferentially reside there by virtue of their continuous expression of the chemokine receptor CXCR5. CD4⁺ T cells expressing CXCR5 have the potential to secrete Th1, Th2 or Th17 cytokines. Therefore, it is unclear whether T_{FH} cells are a distinct subset or rather a ‘chameleon’ state of other subsets that are imprinted by follicular location. T_{FH} cells produce large amounts of IL-21, which acts in an autocrine manner together with IL-6 on their differentiation and expansion, a process that also depends on the Bcl-6 transcription factor.

A population of IL-9 producing cells, derived from Th2 cells by treatment with TGF-β, has also been described. IL-9 was once considered a Th2 cytokine but is

now recognized as not being expressed with IL-4, IL-5 or IL-13. As this population has only been examined *in vitro*, it is unclear whether IL-9 producers should be considered a new subset, to be called Th9, or whether expression of this cytokine reflects adaptation of Th2 cells to a change in the microenvironment in the course of a response triggered by a pathogen or allergen.

More recently, human, but not mouse, Th22 T cells were described (Trifari *et al.*, 2009) and may represent a skin-homing subset responsible for skin inflammation such as psoriasis.

1.4.4 B lymphocytes

B cell development

B cells are defined by their production of immunoglobulin. The B cell matures in the bone marrow, but during fetal life this occurs in the liver. During maturation of the B cell, a series of DNA rearrangements of immunoglobulin heavy-chain genes and light-chain genes occurs for production of membrane-bound and secreted immunoglobulin molecules. As the pre-B cell matures, it acquires μ -chain gene rearrangement together with surrogate invariant light chains ($\lambda 5$ and V pre-B) necessary for effective transport of μ to the cell surface expressed as the pre-B cell receptor. Association of the pre-B cell receptor with $Ig\alpha$ and $Ig\beta$ provides a means for signal transduction that facilitates continued maturation of the pre-B cell kappa (κ) or lambda (λ) gene rearrangements, ultimately resulting in the expression of a complete IgM molecule on the cell surface. Mature B cells co-express surface IgM and IgD after heavy-chain mRNA splicing. All these maturation processes are antigen independent. Thus, activation of mature B cells into immunoglobulin-secreting B cells or long-lived memory B cells and final differentiation into plasma cells depend on antigen interaction.

Similar to events in the thymus with T cells, the maturation and differentiation of B cells is thought to be under the control of cytokines. For example, IL-1 and IL-2 promote B cell activation and growth; IL-4 induces switching to the IgE isotype; IL-5 enhances eosinophils and B cell growth and differentiation; IL-6 increases the rate of secretion of Ig by B cells; and IL-7 promotes proliferation and pre-B cells.

Establishment of the B cell repertoire

Developing B cells follow a program of differential surface antigen expression and sequential heavy- and light-chain gene rearrangement (recently reviewed in LeBien and Tedder, 2008). These processes result in the assembly of the antigen-binding component of the B cell receptor (BCR). Like the TCR, the fully mature BCR also includes additional transmembrane proteins designated $Ig\alpha$ and $Ig\beta$ that activate intracellular signals after receptor binding to antigen. B cells also have a co-receptor complex consisting of CD19, CD81 and CD21 (complement receptor 2) that is activated by binding to the activated complement protein C3d. Both $Ig\alpha$ and $Ig\beta$ have ITAM domains in their cytoplasmic regions and use similar signal

transduction pathways compared with those for T cells. The B cell pathway includes the Src family of kinases, Blk, Fyn and Lyn, which phosphorylate the ITAMs on the Ig α and Ig β chains. The activation signal is then passed through the tyrosine kinase Syk and the B cell linker protein (BLNK) to the downstream signaling components phospholipase C and guanine nucleotide exchange factors. Ultimately, as in T cells, activation of protein kinase C, calcium mobilization and Ras/Rac-dependent activation of mitogen-associated protein kinases leads to activation of new gene transcription that causes cell proliferation and maturation.

Isotype switching and affinity maturation

Naïve B cells express IgM and IgD on their cell surfaces. As B cells mature under the influence of Th cells, T cell-derived cytokines induce isotype switching. Isotype switching is a process of DNA rearrangement mediated in part by the RNA-editing enzyme activation-induced cytidine deaminase, uracil DNA glycosylase, the apurinic/apyrimidinic endonuclease 1 (APE1) and the DNA repair enzyme DNA-PK. Switching moves the rearranged V_HD_HJ_H exon into a position immediately upstream of alternative heavy-chain exons. This permits a functionally rearranged V_HD_HJ_H exon to be used to produce antibodies of different isotypes but the same antigenic specificity. T cell-derived IL-10 causes switching to IgG1 and IgG3, IL-4 and IL-13 cause switching to IgE, and TGF- β causes switching to IgA. IFN- γ or some other undefined product of Th1 cells appears to induce switching to IgG2.

At the same time as B cells undergo isotype switching, an active process produces mutations, apparently randomly, in the antigen-binding portions of the heavy and light chains. This process, designated somatic mutation, also appears to require activation-induced cytidine deaminase, uracil DNA glycosylase, APE1 and DNA repair enzymes. If these mutations result in loss of affinity for the antigen, the cell loses important receptor-mediated growth signals and dies. If, however, the mutations result in increased affinity for the antigen, then the cell producing that antibody has a proliferative advantage in response to antigen and grows to dominate the pool of responding cells. Somatic mutation and clonal expansion of mutated cells occurs in the germinal centres of secondary lymphoid tissues.

T cell-dependent B cell responses

Antigens that activate T cells and B cells establish immunoglobulin responses in which T cells provide 'help' for the B cells to mature. This maturation includes both induction of isotype switching, in which the T cell cytokines control the isotype of immunoglobulin produced, and activation of somatic mutation. The cellular interactions underlying T cell help are driven by the specific antigen and take advantage of the ability of B cells to serve as APCs. B cells that capture their cognate antigen through their membrane immunoglobulins can internalize the antigen and process it intracellularly for presentation on the cell surface in the B cell's class II HLA proteins. Uptake of antigen induces increased class II expression and expression of CD80 and CD86. T cells activated by this combination of

co-stimulator and antigen–class II complex on the B cell then signal reciprocally to the B cell through the interaction of the T cell CD40 ligand with B cell CD40. Isotype switching and somatic mutations are strongly associated with the development of B cell memory. Memory responses, defined as rapid induction of high levels of high-affinity antibody after secondary antigen challenge, are characterized by production of IgG, IgA or IgE antibodies and by somatic mutations in the antigen-binding domains of the heavy and light chains of these antibodies. The development of B-cell memory is critical to the success of vaccination against pathogens and also perpetuates the pathology of many autoimmune and allergic syndromes.

T cell-independent B cell responses

B cells can also be activated successfully without T cell help. T cell-independent B-cell activation occurs without the assistance of T cell co-stimulatory proteins. In the absence of co-stimulators, monomeric antigens are unable to activate B cells. Polymeric antigens with a repeating structure, in contrast, are able to activate B cells, probably because they can cross-link and cluster immunoglobulin molecules on the B cell surface. T cell-independent antigens include bacterial LPS, certain other polymeric polysaccharides and certain polymeric proteins. Somatic mutation does not occur in most T cell-independent antibody responses. Consequently, immune memory to T cell-independent antigens is generally weak. This is why it is difficult to create fully protective vaccines directed against polysaccharide components of microbes.

Bridging innate and adaptive immunity

While most cell types can be easily categorized within the innate and adaptive arms of the immune system, this distinction is less clear for several cell types. This includes B-1 cells, marginal zone (MZ) B cells, certain subsets of $\gamma\delta$ T cells, CD8 $\alpha\alpha$ -expressing T cells in the gut, mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells (Bendelac *et al.*, 2001). Each of these cell types expresses an antigen-specific receptor, either a BCR or TCR, that is generated by V(D)J recombination; however, the repertoire of specificities of these receptors is strongly limited, so that these cells react with a limited diversity of antigens. Hence, the receptors expressed by these so-called innate B and T lymphocytes bear similarities with the PRRs expressed by cells of the innate immune system.

CD1d-dependent iNKT cells are a strikingly conserved subset of T lymphocytes that recognize exogenous and endogenous glycolipid antigens presented by CD1d, and that are implicated in the control of autoimmune, infectious and tumor conditions (Bendelac *et al.*, 2007). iNKT cells are considered innate lymphocytes due to the expression of an effector memory phenotype independent of TCR stimulation by known foreign antigens. iNKT cells (i) homogeneously express the primed-memory CD45R0 and CD44^{high} markers in man and mouse, respectively; (ii) express the differentiation marker CD161, the NK receptor NKR-P1a-c; (iii) rapidly (hours) produce IFN- γ and IL-4 upon activation both *in vivo* and *ex vivo*;

and (iv) are typically CD4⁺ or CD4-CD8. These T cells are unusual in being post-naïve when they leave the thymus of neonates (Smithgall *et al.*, 2008). In older children, iNKT cell have also been shown to exhibit differential responsiveness to milk allergens in those with and without food allergy (Jyonouchi *et al.*, 2011).

MAITs have emerged as a potentially important immunoregulatory cells (Le Bourhis *et al.*, 2011) in the gut and other mucosa covered organs. Like iNKT cells, MAITs are conserved across mammalian species, and they are instructed upon a phylogenetically conserved MHC class I related molecule, MR1, which activates MAIT cells in response to a bacterial challenge (Le Bourhis *et al.*, 2010). MAIT cells are present predominantly in the gut but make up approximately 1–8% of circulating T cells (Martin *et al.*, 2009). In mice, MAIT cells develop only after birth, and this is fully dependent on gut colonization by commensal bacteria (Dusseaux *et al.*, 2011). No studies have yet investigated the relationship of human MAIT cells to commensal colonization or their potential role for the development of (allergic) disease.

In addition to the important advances made in understanding innate cell populations, new insights have been made into innate cell types and cytokines that may be important in the initiation and maintenance of disease. Over the last two years a number of research groups have defined type 2 innate immune responses consisting of epithelial-derived cytokines that can license innate and adaptive immune responses at mucosal sites, including newly described innate helper cells (reviewed in Neill and McKenzie, 2011). These latter cells have been termed NH (natural helper), nuocyte, MPP (multipotent progenitor) and Ih2 cells (innate helper 2) by different groups, and the relationship between them is still unclear. Importantly, they are able to produce considerable quantities of IL-5 and IL-13 in response to the novel cytokines IL-25 and IL-33. These latter cytokines are able to induce Th2-associated pathologies *in vivo* (Fort *et al.*, 2001).

Lymphoid tissue and cellular communication

Cellular interactions are essential for a normally regulated, protective immune response. In particular, T cell help is needed to generate high-affinity antibody with memory against most protein antigens. A major challenge for the immune system of a naïve subject is to bring rare antigen-specific B cells together with rare antigen-specific T cells and antigen-charged APCs. The primary role of the secondary lymphoid tissues is to facilitate these interactions. Generally, the secondary lymphoid organs contain zones enriched for B cells (follicles) and other zones enriched for T cells (Fu and Chaplin, 1999). The B-cell zones contain clusters of follicular DCs that bind antigen–antibody complexes and provide sites adapted to efficient B cell maturation, somatic mutation and selection of high-affinity B cells. The T cell zones contain large numbers of dendritic cells that are potent APCs for T cell activation.

Recruitment of leukocytes both to secondary lymphoid tissues and to peripheral tissue sites of microbial invasion is essential for intact host defense. The traffic of lymphocytes from blood to tissues occurs via a series of lymphocyte–endothelial interactions that are dependent on binding between lymphocyte surface molecules

and endothelial molecules together with an interaction between tissue-specific chemokines and corresponding chemokine receptors on lymphocytes (Sackstein, 2005). The combination of L-selectin (CD62L) and the chemokine receptor CCR7 expressed on naïve T cells allows them to migrate into secondary lymphoid tissues (Sallusto *et al.*, 1999; Campbell *et al.*, 2001). Upon Ag stimulation, naïve T cells get activated and acquire a new profile of tissue-specific homing receptors guiding them to peripheral tissues drained by the lymph node (Campbell and Butcher, 2002). Thus, T cells activated in mesenteric lymph nodes or in Peyer's patches start to express the gut-homing integrin $\alpha 4\beta 7$ that binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is only expressed on high-endothelial venules in gut-associated lymphoid tissues (GALT) and post-capillary venules in the gut (Campbell and Butcher, 2002). T cells activated in cutaneous lymph nodes instead commence to express cutaneous lymphocyte-associated Ag that mediates localization to the skin via interaction with vascular ligand E-selectin (Campbell and Butcher, 2002, Dudda and Martin, 2004). Moreover the chemokine receptor CCR9 directs T cells to the small intestine, while CCR4 seems to attract T cells to non-gastrointestinal tissues, such as the skin and the lung (Kunkel *et al.*, 2000).

Tolerance and immunoregulation

CD4⁺ regulatory T cells (Treg) suppress effector T cells and prevent or limit reactivity to self-antigens (Sakaguchi, 2005) and some pathogens (Suvas *et al.*, 2003), to blunt inflammation and to maintain antigen-specific T-cell homeostasis (O'Garra and Vieira, 2004). Multiple Treg cell populations have been reported (O'Garra and Vieira, 2004), with natural Treg cells being the best characterized. Natural Treg cells constitutively express CD25 (IL-2-receptor α -chain) (Sakaguchi *et al.*, 1995), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) (Takahashi *et al.*, 2000) and the forkhead/winged helix transcription factor Foxp3 (Fontenot *et al.*, 2003), which is a key control gene in their development and function. Although no single marker uniquely identifies Treg cell populations, panels of markers allow identification of CD4⁺ T cells with suppressive activity (Sakaguchi *et al.*, 2010). Treg cells are often defined as CD3⁺CD4⁺CD25⁺FoxP3⁺ cells. The expression of intracellular CTLA-4 and Foxp3 is positively correlated with surface CD25 expression. Higher levels of intracellular Foxp3 and CTLA-4 and lower levels of surface CD127 consistently distinguish CD25^{hi} cells from CD25^{int/low} and CD25⁻ cells. More recent studies demonstrated that Treg cells also express low level of CD127 marker (IL-7 receptor) (Hartigan-O'Connor *et al.*, 2007) and are negative for CD49d (α -chain of integrin VLA-4; $\alpha 4\beta 1$) (Kleynietfeld *et al.*, 2009).

In addition to CD4⁺CD25⁺Foxp3⁺ Treg cells, it has been shown that other T cells also possess regulatory activity. Most of these cells – such as IL-10-secreting T regulatory type 1 (Tr1) cells, TGF β -secreting T helper 3 (Th3) cells and certain CD4⁺CD8⁻ T cells and CD8⁺CD28⁻ T cells – are adaptively regulatory: that is, they acquire regulatory functions following specific antigenic stimulation in particular cytokine milieus. They therefore contrast with naturally occurring CD4⁺Foxp3⁺ Treg cells, most of which are developmentally determined in the thymus as a

distinct T cell subpopulation that is specialized for suppressive function (recently reviewed in Sakaguchi *et al.*, 2010).

Recently, there has been a developing interest in the role of the kynurenines in immune function and regulation. The kynurenine pathway is the major route for tryptophan (TRY) metabolism in mammals. In the first step of this metabolic process, TRY is transformed to kynurenine (KYN) in reaction catalyzed by two dioxygenase enzymes: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). IDO transforms tryptophan into kynurenine, which is then metabolized into other catabolites through the action of enzymes within the kynurenine pathway. Of note, IDO occupies a key position connecting the immune system and the kynurenine pathway. There are accumulating lines of evidence now highlighting the immunosuppressive effect of this enzyme (reviewed in Mandi and Vecsei, 2011). Inhibition of T cell function, activation of Tregs and inhibition of NK cells are among the important immunosuppressive effects of IDO and kynurenines.

Recent studies have demonstrated a complex and crucial role of IDO in immunoregulation during infection, pregnancy, allergy, autoimmunity, transplantation and neoplasia (Mellor and Munn, 2004; Pallotta *et al.*, 2011). Interest continues to focus on the role of kynurenines in the immune system.

Epigenetic and microRNA regulation

In recent years, there has been an increased appreciation of the levels of complexity inherent within the immune system. MicroRNAs (miRNAs) and epigenetic mechanisms have both been highlighted as important regulators of many biological systems, including the innate and adaptive immune system.

Epigenetic mechanisms have been shown to regulate Th1, Th2 differentiation (Fields *et al.*, 2002; Lee *et al.*, 2002, 2006; Santangelo *et al.*, 2002; Shin *et al.*, 2005) and Treg differentiation (Janson *et al.*, 2008; Polansky *et al.*, 2008). It has also been shown that miRNAs have unique expression profiles in cells of the innate and adaptive immune systems and have pivotal roles in the regulation of development and function of such cells (O'Connell *et al.*, 2010). Genome-wide analysis of miRNA has been made for total human CD4⁺ and CD8⁺ T cells (Landgraf *et al.*, 2007). More recently, a broad analysis of miRNA expression in 17 different highly purified human lymphocyte subsets was performed and specific miRNA signatures identified. Of interest, the authors focused on naive CD4⁺ T-cells and found that miR-125b regulated a network of genes encoding molecules involved in CD4⁺ T cell differentiation, including cytokine receptors, cytokines and transcription factors (Rossi *et al.*, 2011).

1.5 Conclusion

The recent discoveries in the field of innate immunity have significantly advanced our understanding of the highly complex and intricate set of interactions between innate and adaptive immune systems. Also, we now have a better appreciation for

the level of integration of the two systems (see the recent review by Pulendran *et al.*, 2010).

1.6 References

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2

Mucosal immunity in a healthy gut

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Abstract: The intestinal immune system has generated two arms of adaptive anti-inflammatory defense which normally preserve the epithelial barrier: (i) immune exclusion performed by secretory IgA (SIgA) (and SIgM) antibodies to control surface colonization of micro-organisms and dampen penetration of potentially harmful antigens; and (ii) suppressive mechanisms to avoid hypersensitivity to innocuous antigens, particularly food proteins and the commensal microbiota. The latter phenomenon ('oral tolerance') largely depends on regulatory T (Treg) cells induced in mesenteric lymph nodes to which mucosal dendritic cells carry exogenous antigens and become conditioned for stimulation of Treg cells. Polymeric Ig receptor (pIgR/SC) knock-out mice that lack SIgA and SIgM show decreased epithelial barrier function and increased uptake of antigens from food and commensal bacteria. They therefore have a hyper-reactive immune system which is counteracted by enhanced intestinal tolerance induction as a homeostatic back-up mechanism.

Key words: epithelial barrier, oral tolerance, pattern-recognition receptors, microbe-associated molecular patterns, secretory IgA, polymeric Ig receptor, IgG, IgE, food allergy, inflammatory bowel disease.

2.1 Introduction

Mammalian host defenses have successfully handled environmental confrontations for millions of years. To this end, numerous genes involved in innate and adaptive immunity have been subjected to evolutionary modifications, thus being shaped according to microbial pressures and exogenous (including dietary) impacts. This modulation has been influenced by various ways of living such as hunting, fishing, gathering, agriculture and animal husbandry. A good example of the interplay between genes and the environment is wheat gluten-induced enteropathy (celiac disease) which has a relatively high (but variable) prevalence in

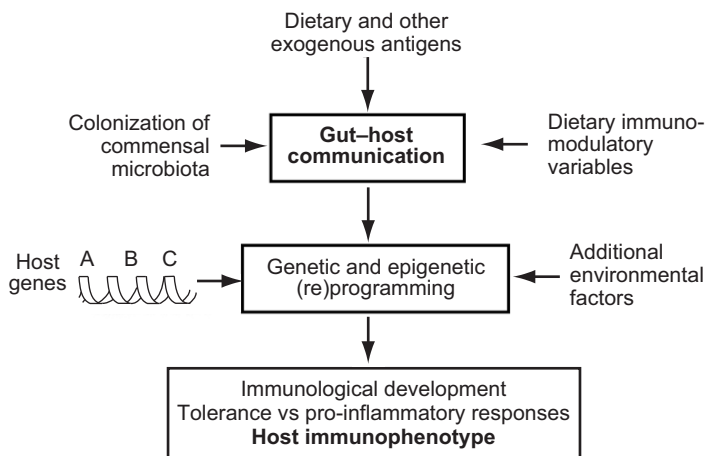


Fig. 2.1 Influence of exogenous variables on gut–host communication and immunological development. The gut represents a large contact organ that transmits biological signals from the environment to the host. These signals are essential for development of immunity and may determine the balance between tolerance and pro-inflammatory responses. The interaction of exogenous factors with host genes (A, B, C, etc.) provides opportunities not only for programming but also for reprogramming of the immune system and may be influenced by epigenetic variables. The immunophenotype of the host may therefore be determined at various levels, partly through epigenetic regulation.

modern societies.¹ This may partly reflect past genetic selection for enhanced immunity against the new infectious diseases (e.g. zoonoses), whose appearance accompanied the development of farming, domestication of animals and greater crowding together of people. Such selection may have resulted in human leukocyte antigen (HLA) polymorphisms that increase immune reactivity to gluten. Although classical celiac disease depends on gluten-activated CD4⁺ effector T cells in a HLA-DQ2- (or HLA-DQ8-) restricted manner, there are clearly other genetic risk factors and additional exogenous triggers such as viral infections. It seems that environmental variables may compensate for genetic risk factors other than HLA in the pathogenesis.¹ Thus, the effector mechanisms and the disease outcome may develop in several ways, and this is probably true also for other hypersensitivity reactions to food which has been introduced into the diet relatively recently.

The major arena for the complex interactions between genes and the exogenous impact is the gut. This is therefore the most crucial organ for communication between the environment and the body (Fig. 2.1). In an adult human being, the gut epithelium covers a surface area of perhaps 300 m² when villi, microvilli, crypts and folds are taken into account. This barrier has generally only one cell layer and is therefore vulnerable but normally well protected by numerous chemical and physical innate defense mechanisms which cooperate intimately with a local adaptive immune system.² The dominating component of the latter is an immunoglobulin A (IgA)-generating B-cell population which basically provides an anti-inflammatory first-line defense by mainly giving rise to secretory IgA (SIgA)

antibodies performing ‘immune exclusion’.^{3,4} This term is coined for low- and high-affinity antibody functions at the mucosal surface, aiming to control both microbial colonization and penetration of noxious antigens through the epithelial barrier.^{5–7}

The formation of SIgA depends on IgA-producing plasma cells (PCs) and their immediate precursors (plasmablasts) which accumulate in the mucosa by selective homing mechanisms after priming of B cells in gut-associated lymphoid tissue (GALT) – including Peyer’s patches, isolated lymphoid follicles (ILFs) and the appendix.^{3, 8} At least 80% of the body’s Ig-producing cells are located in gut mucosa, which therefore constitutes the body’s largest effector organ of humoral immunity.⁹ In addition, hypersensitivity due to excessive penetration of exogenous antigens into the mucosa is normally counteracted by adaptive hyporesponsiveness to innocuous agents. This phenomenon is traditionally referred to as ‘oral tolerance’ when induced via the gut;¹⁰ it inhibits in particular over-reaction to dietary proteins and components of the commensal microbiota.^{2,3,11}

Such mucosally induced hyporesponsiveness seems to be a rather robust adaptive immune function in view of the fact that large amounts of food proteins pass through the gut, while overt and persistent food allergy is not so common. Moreover, most individuals live in harmony with a gut microbiota consisting of commensal bacteria amounting to ten times the number of cells of the body. This homeostatic mutualism is regulated by specialized dendritic cells (DCs) that are ‘decision makers’ of the immune system when they perform their HLA-dependent antigen-presenting function, thus linking innate and adaptive immunity by sensing the exogenous impact. A balanced indigenous microbiota is required to drive the normal development of both mucosal lymphoid tissue, the epithelial barrier with its SIgA (and SIgM) system, and oral tolerance – including the generation of regulatory T (Treg) cells.

As the mucosal induction and tuning of all these homeostatic mechanisms depend on immunological stimuli, the neonatal period is particularly critical. The mucosal barrier and its reinforcement by SIgA, as well as the immunoregulatory network, require both adaptive and innate induction by antigens and conserved microbe-associated molecular patterns (MAMPs) – the latter activating cellular pattern-recognition receptors (PRRs) sensing the exogenous impact on DCs and other cells.^{2,12–14} It is still not clear how these receptors can discriminate between signals provided by MAMPs from commensals and MAMPs from pathogens (previously called pathogen-associated molecular patterns, PAMPs); but this distinction is clearly required to elicit a tolerogenic *versus* a productive immune response that adequately combats an infectious agent. Various scenarios may be visualized – the most likely being that overt pathogens, in addition to signaling through PRRs, exhibit special danger signals related to the pathogenicity itself such as factors determining virulence and invasiveness.¹⁵

The healthy gut harbors a huge collection of ‘kind’ or beneficial bacteria (‘symbionts’) that constitute some ten times the number of cells in the body, perhaps amounting to 10^{13} – 10^{14} microbial cells and a total weight of 1–2 kg.¹² The intestinal immune adaptation to ordinary dietary antigens is also remarkably

successful in view of the fact that a ton of food may pass through the gut of an adult human being every year, usually without causing adverse reactions. Classical food allergy and inflammatory bowel disease (IBD) reflect lack of such homeostasis, either due to retarded immunological development and immaturity of the mucosal barrier with its innate and adaptive defense, abrogation of the epithelial barrier function or a persistently imbalanced immunoregulatory network – probably on a polygenic susceptibility background as alluded to above.

The mainstream theory explaining the rise observed for allergies and other immune-mediated disorders in affluent societies,¹⁶ is based on the extended hygiene hypothesis, underscoring an important role of changes in the environmental microbial impact on the immune system.¹⁷ In essence, the idea is that industrialized societies, by modern measures introduced over the last decades, have deprived the infants of adequate immunological stimuli.¹⁸ The relations between a westernized lifestyle and the origins of human disease comprise hygienic, dietary and medical practices that have altered the pattern of microbial exposure, including the composition of the gut microbiota. Therefore, research on microbial–host interactions aims to reverse hypersensitivity by promoting tolerance.^{14,19,20}

For effective strategies to prevent immune-mediated diseases, it is essential to understand how exogenous variables influence the adaptive immunological programming in health and how the effector mechanisms are regulated in a homeostatic fashion. The scientific basis of such efforts will be discussed in this chapter.

2.2 Induction of adaptive immunity in the gut

2.2.1 Homeostasis-promoting mechanisms

Numerous genes regulate the innate and adaptive arms of the immune system. Human immunogenetics has evolved to identify ‘danger’ under the pressure of a ‘dirty environment’, even long beyond the hunting-gathering period. In this evolutionary process the intestinal immune system has generated its two adaptive anti-inflammatory strategies: immune exclusion performed by SIgA to control surface colonization of micro-organisms and to inhibit mucosal penetration of potentially dangerous agents; and oral tolerance to avoid local and peripheral hypersensitivity against innocuous antigens which have crossed the epithelial barrier (Fig. 2.2). Together, the two strategies apparently explain why overt and persistent problems with gut immunopathology are relatively rare. Remarkably, the suppressive mechanisms usually operate successfully in face of the enormous commensal microbiota separated from the internal body milieu only by the monolayered epithelium with its mucus coat.^{12,13,21,22} Notably, however, immunoregulatory differences apparently exist between humans and clean laboratory mice due to a more strict shielding of gut bacteria from the systemic immune system in the rodent species, normally leading to a state of immunological ignorance of the commensal microbiota.^{12,13,23–26}

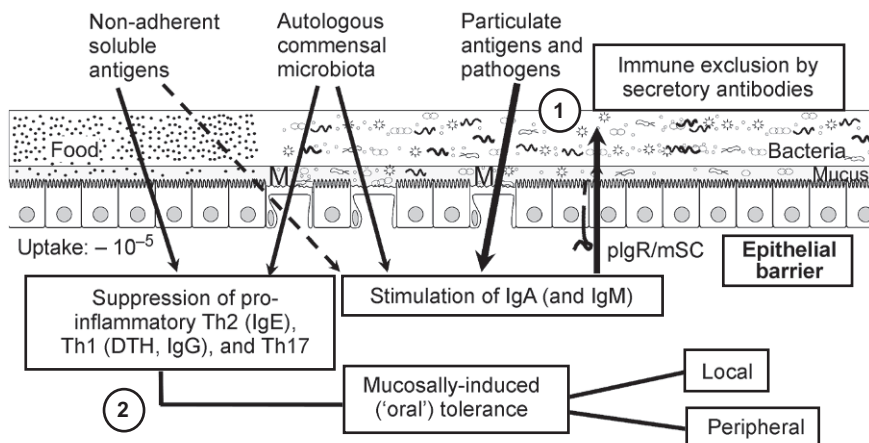


Fig. 2.2 Two levels of mucosal anti-inflammatory immune defense aiming at preserved integrity of the epithelial barrier: (1) Productive immunity providing immune exclusion to limit microbial colonization and penetration of harmful agents. This first line of defense is principally mediated by secretory antibodies of the IgA (and IgM) class in cooperation with various innate defense mechanisms (not shown). Secretory antibodies are exported by the epithelial polymeric Ig receptor (pIgR), also called membrane secretory component (mSC), after its apical cleavage. Secretory immunity is preferentially stimulated by pathogens and other particulate antigens taken up through specialized M cells (M) located in the dome epithelium covering inductive mucosa-associated lymphoid tissue (see Fig. 2.3). (2) Innocuous soluble antigens (e.g. food proteins; magnitude of uptake after a meal is indicated) and the commensal microbiota are also stimulatory for secretory immunity, but induce additionally suppression of pro-inflammatory Th2-dependent responses (IgE antibodies), Th1-dependent delayed-type hypersensitivity (DTH), IgG antibodies, and Th17-dependent neutrophilic reactions (graded arrows indicate presumed importance of stimulatory pathways). This Th-cell balance is regulated by a complex mucosally-induced phenomenon called 'oral tolerance' in the gut, in which regulatory T cells are important (see Figs 2.10 and 2.12). Their suppressive effects can be observed both locally and in the periphery.

As alluded to above, oral tolerance appears as a robust adaptive immune function because even healthy adults absorb measurable concentrations of intact food antigens, particularly after meals – corresponding to 10^{-5} of the intake and reaching a circulating level of 3–10 ng/ml²⁷ – thus representing a small fraction of the total daily protein uptake which perhaps amounts to 130–190 g.²⁸ The epithelial tightness and the immunoregulatory network remain fragile for a variable period after birth.^{29,30} Importantly, animal experiments show that the postnatal development of mucosal homeostasis depends on the establishment of a balanced commensal microbiota as well as adequate timing and dosing of dietary antigens when first introduced.^{10,12,22,30} The effect of the indigenous bacteria on the postnatal GALT development is strikingly revealed in experimental animals colonized with a conventional microbiota after being reared in a germ-free state.^{31,32} Moreover, food proteins apparently also contribute to the development of GALT and the

intestinal immune effector compartments, as observed in mice reared on a diet containing casein compared with a balanced amino acid-based diet.³³

2.2.2 Gut-associated lymphoid tissue

Of all the organized mucosa-associated lymphoid tissue (MALT) of the body GALT predominates, particularly the Peyer's patches (mainly in the distal ileum), and the numerous ILFs (particularly in the large bowel).^{8,34} All MALT structures represent inductive sites for mucosal immune responses in a regionalized manner depending on the homing molecules expressed on the activated T and B cells (Figs 2.3 and 2.4). Thus, in the gut the principal effector sites are the lamina propria and epithelium, but these compartments may nevertheless contribute to retention, proliferation and differentiation of immune cells.^{8,35}

Although Peyer's patches are present at birth, it takes some time before they become activated as signified by germinal centers, and the induction of ILF organogenesis depends on postnatal exogenous stimuli, at least in mice.^{8,36} The B-cell follicles of GALT are covered by a specialized epithelium containing very thin 'microfold' or membrane (M) cells which, together with intraepithelial DCs, transport antigens from the gut lumen into the lymphoid tissue.³⁷

Peyer's patches resemble lymph nodes with inter-follicular T-cell zones and a variety of antigen-presenting cells (APCs) such as DCs and macrophages, whereas ILFs have a more sparse T-cell zone. All MALT structures are devoid of encapsulation and contain no afferent lymphatics;⁸ their supply of antigens depends exclusively on sampling directly from the mucosal surface through the specialized follicle-associated epithelium (Fig. 2.3). Induction and regulation of intestinal immunity hence takes place primarily in GALT and the draining mesenteric lymph nodes (MLNs), while terminal differentiation of B cells to plasmablasts and PCs occurs in the lamina propria where secondary T-cell signals are generated when antigens are presented by local DCs and macrophages.³⁵

Naïve (inexperienced) T and B cells enter MALT/GALT (and also lymph nodes) via specialized postcapillary or so-called high endothelial venules (HEVs) (Fig. 2.3). After being activated to become memory/effector T and B cells, they migrate through regional lymph nodes (e.g., MLNs) and further on via lymph and peripheral blood for subsequent extravasation at mucosal effector sites such as the gut lamina propria. This process is directed by the profile of adhesion molecules and chemokines expressed on the microvasculature, the endothelial cells thus exerting a local 'gatekeeper function' for mucosal immunity. The lamina propria is schematically depicted in Fig. 2.3 with its immune cells, including B cells, J chain-expressing (+ J) IgA⁺ and IgM⁺ PCs, IgG⁺ PCs with a variable J-chain level [(J)], and CD4⁺ T cells with effector (Teff) or regulatory (Treg) function. Additional features are the generation of SIgA and secretory IgM (SIgM) via receptor-mediated epithelial export, as well as paracellular leakage of smaller amounts (broken arrow) of both locally produced and serum-derived IgG antibodies into the lumen. Note that IgG cannot interact with J chain to form a binding site for the polymeric Ig receptor (pIgR). Also indicated in the figure is the distribution of intraepithelial lymphocytes

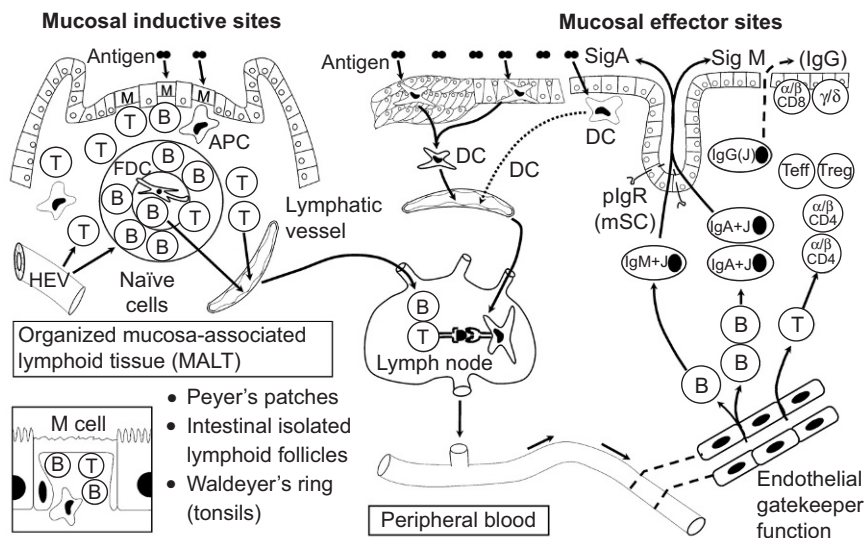


Fig. 2.3 Compartments, cells and functions of the human mucosal immune system. Inductive sites for mucosal T and B cells are the regional mucosa-associated lymphoid tissue (MALT) with its B-cell follicles and M cell (M)-containing follicle-associated epithelium (FAE) through which exogenous antigens are transported to antigen presenting cells (APCs), including dendritic cells (DCs), macrophages, B cells and follicular dendritic cells (FDCs) in germinal centers. Naïve T and B cells enter MALT via high endothelial venules (HEVs). Insert (lower left) shows details of FAE with an M cell and its 'pocket' containing various cell types. In addition, intra- or subepithelial DCs may capture antigens and migrate via draining lymph to regional lymph nodes where they become active APCs, which stimulate T cells for productive (Teff) or suppressive (Treg) immune responses. Also indicated is the generation of secretory IgA (SigA) and SIgM via receptor-mediated epithelial export of locally produced J chain-containing dimers/polymers of IgA and pentamers of IgM, whereas IgG may leak out by the paracellular route. The polymeric Ig receptor (pIgR) is also known as membrane secretory component (mSC).

(mainly T-cell receptor α/β^+CD8^+ and some γ/δ^+ T cells), constituting a frontline cellular defense. Importantly, animal experiments have shown that oral tolerance resulting from induction/expansion of Treg cells can occur in the absence of GALT, thus being dependent on antigen transport to MLNs from gut mucosa through draining lymph by specialized DCs (Fig. 2.3), as discussed later.^{38,39}

2.2.3 Induction and homing of intestinal immune cells

Antigens are presented to T cells in GALT and MLNs by APCs after intracellular processing.² The activated helper T (Th) cells release mediators (cytokines), and especially transforming growth factor (TGF)- β induces the switch of B cells from surface membrane expression of IgM to IgA in GALT follicles.^{3,8,35} Memory/effector T and B cells emerging from this activation process migrate rapidly via lymphatics to MLNs where some of the effector B cells may be further differentiated to plasmablasts. Most activated GALT-derived immune cells will reach

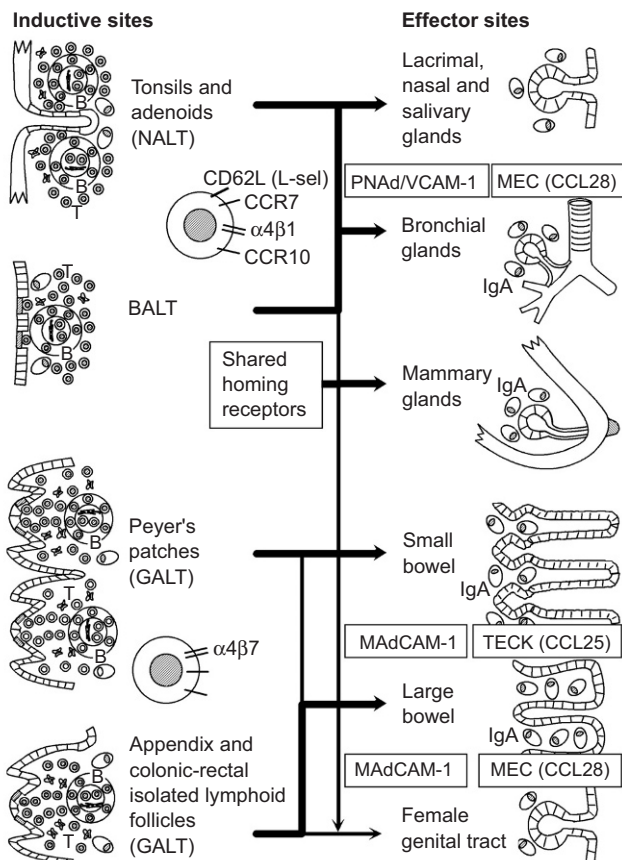


Fig. 2.4 Model for homing of activated B cells from mucosal inductive sites to secretory effector sites in the integrated human mucosal immune system. Compartmentalization in the trafficking is indicated – the heavier arrows representing preferential B-cell migration pathways. Homing from gut-associated lymphoid tissue (GALT) is determined mainly by integrin $\alpha 4 \beta 7$ on the activated cells, interacting with MAdCAM-1 expressed on the microvascular endothelium in the intestinal lamina propria ('gatekeeper' function, cf. Fig. 2.3). In the small intestine, attraction and/or retention of CCR9-expressing cells is additionally mediated by the chemokine CCL25/TECK, while CCL28/MEC interacting with CCR10 is mainly active in the large bowel. Other adhesion molecules, such as CD62L/L-selectin (L-sel) and $\alpha 4 \beta 1$ that bind to endothelial PNAd and VCAM-1, respectively, are employed mainly by B cells primed in nasopharynx-associated (NALT) and bronchus-associated (BALT) lymphoid tissue. Human NALT comprises the various lymphoepithelial structures of Waldeyer's ring, including the nasopharyngeal tonsil (adenoids) and palatine tonsils. In this region, abundantly produced epithelial CCL28/MEC attracts activated B cells via CCR10. The female genital tract (cervix mucosa) may employ similar molecular homing mechanisms as the upper aerodigestive tract and the large bowel – therefore probably receiving activated B cells from inductive sites in both these regions. Also lactating mammary glands appear, by shared homing mechanisms, to receive primed cells from NALT as well as GALT – and much more efficiently so than the female genital tract. Expression of CD62L and CCR7 on NALT-derived cells allows integration with the systemic immune system.

peripheral blood via the thoracic duct and finally become selectively seeded into the lamina propria (Fig. 2.3).

The extravasation of effector/memory T and B cells at secretory effector sites is facilitated by compartmentalized homing receptors interacting with ligands (addressins) on the microvascular endothelium, while additional fine-tuned navigation is conducted by chemokines. Such B-cell homing from GALT targets particularly the intestinal lamina propria but also, to some extent, distant secretory mucosae and glandular sites (Fig. 2.4) – notably the lactating mammary glands.⁴⁰ The extent of B-cell retention and terminal differentiation to plasmablasts and PCs in the gut lamina propria depends on the local intensity of secondary signals, provided by chemokines and APC-processed antigens via activated CD4⁺ Th cells and their cytokines.³⁵

Retinoic acid (RA) derived from vitamin A in the diet is important for the expression of gut-homing molecules on T and B cells when they are activated in GALT and during their transit through MLNs.⁴¹ RA also exerts a positive impact on the differentiation of naïve B cells arriving in GALT by enhancing the heavy-chain switching to IgA both in humans and mice,^{41,42} as well as inducing the expression of the gut-homing integrin $\alpha 4\beta 7$ and the CC chemokine receptor CCR9 (Fig. 2.4). The phenotype of APCs in GALT, which seems to be imprinted by the action of gut bacteria on cellular PRRs, promotes RA generation by the APC expression of retinaldehyde dehydrogenase (RALDH); and the expression of inducible nitric oxide synthase (iNOS) enhances, via nitric oxide (NO), the release of innate switch factors which are cytokine members of the tumor necrosis factor (TNF) family, namely APRIL (a proliferation inducing ligand) and BAFF/BlyS (B cell-activating factor of the TNF family/B lymphocyte stimulator),^{8,42} as well as the release of the activated IgA switch factor TGF- β from Th cells.⁴³ Furthermore, in mice it has been shown that a fraction of these follicular T helper (T_{FH}) cells may be derived from Treg cells.⁴⁴ There may thus be a cellular link between intestinal IgA and mucosal tolerance induction, for which TGF- β is likewise important.²⁶ Also interleukin (IL)-10, that contributes to terminal differentiation of IgA⁺ PCs together with IL-6, is an important cytokine in oral tolerance, as discussed later.

The propensity of the mucosal immune system to generate low-affinity cross-reactive background antibodies is probably explained by the extensive innate drive imposed on it by MAMPs derived from the abundant commensal microbiota interacting with PRRs.^{8,45} Thus, experiments have revealed a role of the important PRRs designated Toll-like receptors (TLRs) for B-cell differentiation in GALT.^{8,45} Interestingly, human GALT follicles contain the apparatus to support both T cell-dependent and T cell-independent (not involving CD40–CD40L/CD154 interactions) class switch recombination (CSR) pathways to IgA.^{46,47} This has been documented by showing GALT-restricted human expression of activation-induced cytidine deaminase (AID) – an essential enzyme for CSR to take place. Also notable, the T cell-independent switch factor APRIL, and its receptors TACI (transmembrane activator and CAMEL interaction) and BCMA (B cell maturation antigen) are expressed both in human GALT and human intestinal mucosa but, importantly, there is no co-expression of these receptors and AID beyond the GALT structures.⁴⁶

2.2.4 Different pathways to intestinal IgA production

Previous claims about B-cell switch to IgA in human lamina propria seem questionable,⁴⁶ and the same is true for an extrafollicular subclass switch from IgA1 to IgA2 which has also been proposed.⁴² The possibility remains, however, that the unique T cell-independent B1 cell population generated in the mouse omentum⁴⁸ may provide a substantial fraction of lamina propria PCs and be subjected to IgA switch either in the peritoneal cavity or in the lamina propria of this species.^{49,50} However, recent evidence suggests that T cell-independent CSR in the mouse gut does depend on GALT structures although not on germinal centers.⁵¹ Thus, a likely possibility is that T cell-independent switch to IgA in the mouse mainly takes place in the numerous ILFs present in the distal gut,^{8,52} and that APRIL outside of GALT structures both in mice and humans mainly promotes the survival of PCs in the mucosa.⁵³ There may be several cellular sources of APRIL in the gut, such as epithelial cells, neutrophils and eosinophils. A recent study showed that eosinophils in mouse bone marrow supported PC survival by secreting APRIL and IL-6.⁵⁴

Finally, the long-lasting debate about the role of vasoactive intestinal polypeptide (VIP) in IgA induction seems to be clarified.³ An IgA-inducing protein (IGIP) first identified in the bovine species has now been characterized in humans – with somewhat different properties;⁵⁵ its CD40L/CD154-stimulated expression in DCs was found to be 35-fold enhanced by VIP, and IGIP was directly shown to induce CSR to IgA in naïve (surface IgM⁺IgD⁺) B cells.

A problem with all the experimental studies on IgA-promoting factors operating in GALT, such as TGF- β , RA and IGIP, is that they have not been tested for J chain-inducing properties. The small polypeptide named ‘joining’ (J) chain is a prerequisite for the production of dimeric IgA and its binding to the epithelial pIgR, as discussed later. Although there is considerable information about the regulation of J-chain expression in mice, such knowledge is lacking in humans; and the factors responsible for the high level of J chain in the GALT-derived B-cell lineage are not known in any species.⁵⁶ However, this biological feature seems to be associated with the propensity of IgA switching in GALT (Fig. 2.5); the question remains whether gut-related factors such as RA or VIP may be involved in the extensive drive for IgA and J-chain expression, or whether it reflects a swift clonal B-cell turnover in GALT caused by the continuous exposure to novel microbial epitopes in the gut.³⁵

2.2.5 Intestinal immunity during infancy

Only a few IgA⁺ plasmablasts circulate in the blood of newborns (<8 per million mononuclear cells), although this number is remarkably increased (~600 per million mononuclear cells) already after one month, mainly reflecting the progressive microbial stimulation of GALT.⁵⁷ An initial early elevation of circulating plasmablasts (mainly IgM⁺) occurs in preterm infants, especially those with intrauterine infections.⁵⁸ Thus, mucosal immune cells are competent at least during the final trimester, but APCs need to be activated by microbial factors that enable

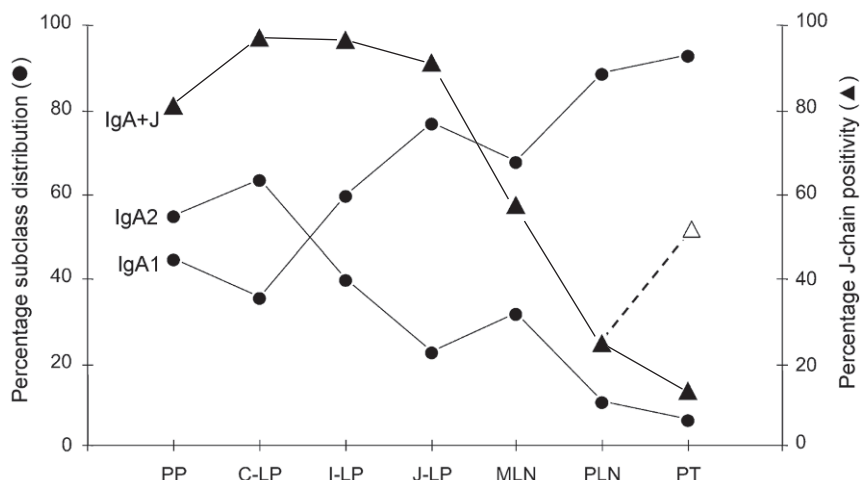


Fig. 2.5 Subclass distribution and J-chain expression of IgA-producing plasma cells in various normal extrafollicular tissue compartments from adults. PP, Peyer's patch; C-LP, I-LP and J-LP, colonic, ileal and jejunal lamina propria, respectively; MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes; and PT, palatine tonsils. For the latter organ, J-chain data from children who had never experienced recurrent tonsillitis are also indicated (open triangle connected by dashed line). Based on published immunohistochemical data from the author's laboratory.

them to provide appropriate co-stimulatory signals to naïve T cells.⁵⁹ The commensal microbiota is important to this end as shown by the fact that the number of intestinal IgA⁺ PCs is normalized four weeks after exposure of germ-free mice to a conventional gut microbiota.^{60,61} *Bacteroides* and *Escherichia coli* strains seem to be particularly immunostimulatory, but also lactic acid-producing bacteria contribute.^{62,63}

The retarded postnatal activation of GALT parallels the functionally decreased systemic immunocompetence in the newborn period.^{29,30,59,64} Thus, peripheral CD4⁺ Th cells of infants show reduced capacity for cytokine production and B-cell help. One reason might be that there are relatively few circulating memory (CD45R0⁺) T cells in infancy, although the responsiveness of neonatal naïve (CD45RA⁺) T cells does not differ significantly from that of virgin counterparts in adults. Indeed, the chief explanation for the immunological immaturity in infancy appears to be a deficient APC function.⁶⁵

2.3 Secretory immunity reinforcing the mucosal barrier

2.3.1 Production of secretory antibodies

Most mucosal PCs produce dimers and larger polymers of IgA (collectively called pIgA), which contain the 15 kD disulfide-linked J chain (Fig. 2.6). This polypeptide is a prerequisite for the dimerization and the active export of pIgA through

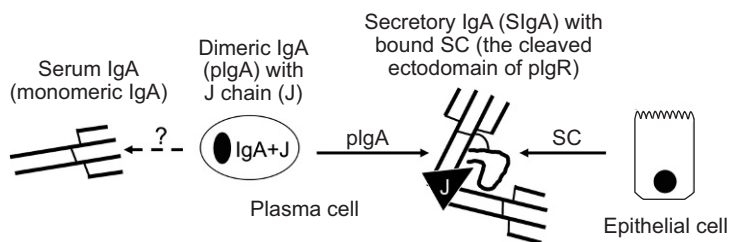


Fig. 2.6 Secretory IgA (SIgA) generation. Depiction of how SIgA is formed as a hybrid antibody molecule stabilized by a disulfide bridge between the two cell products, pIgA and secretory component (SC). The amount of pIgA produced by a plasma cell depends on its level of J-chain expression, which generally is high in mucosal and glandular tissue, particularly in the gut. IgA produced by the systemic immune system is mainly monomeric. Some features of the SIgA system are: more IgA is produced every day than all other antibody classes collectively (IgG, IgM, IgD, IgE); daily adult intestinal SIgA export, >40 mg/kg (~3 g/day); determination of specific SIgA is the best 'readout' for mucosal immunity although afflicted with many pitfalls.

secretory epithelia such as the intestinal crypts.^{66–68} The transport is mediated by pIgR, which is also known as membrane secretory component (SC).³⁴ J chain-containing pentameric IgM is externally transported by the same mechanism.^{3,66,69}

Apical proteolytic cleavage of the extracellular portion of pIgR enables release of SIgA and SIgM to the lumen (Fig. 2.7). In this manner, the ectodomain of pIgR is 'sacrificed' to become bound SC which stabilizes the quaternary structure of the secretory antibodies, particularly SIgA.^{3,35} Unoccupied pIgR is constitutively exported in the same manner; the cleaved ectodomain is called free SC³⁴ and exhibits certain innate immune properties such as affinity for *E. coli* and *Clostridium difficile* toxin.⁴ Bound SC confers such properties to SIgA and contributes to its mucus-binding capacity and its trapping of bacteria in biofilms.^{4,70}

Immune exclusion performed by SIgA and SIgM thus depends on an intimate cooperation between the mucosal B-cell system and the pIgR-expressing epithelium (Fig. 2.7). Serum-derived and locally produced IgG antibodies may also to some extent contribute to immune exclusion when reaching the lumen by non-injurious paracellular diffusion⁷¹ or after being transported by the neonatal Fc receptor, FcRn.⁷² However, IgG is rapidly degraded in the gut lumen, although the hepatic superantigen (protein Fv) may form large complexes with degraded antibodies of different specificities, thereby reinforcing their immune exclusion function.⁷³

As mentioned above, only small amounts of pentameric IgM are normally exported by pIgR (Fig. 2.7). SIgM is not as stable as SIgA because bound SC is covalently stabilized only in the latter.⁶⁹ Immune exclusion is therefore normally performed mainly by SIgA in cooperation with innate defenses such as mucus, defensins and peristalsis.^{2,45} In newborns and subjects with selective IgA deficiency, however, SIgM antibodies are of greater importance than in healthy adults.^{29,35} Thus, IgA is generally undetectable in the mucosa before 10 days of age,

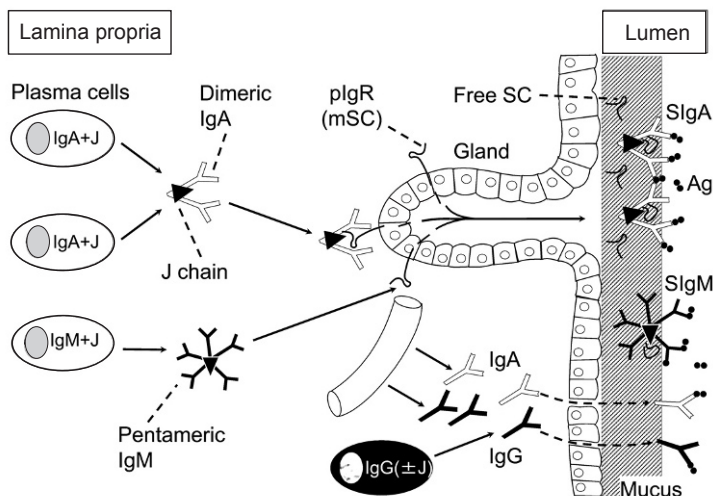


Fig. 2.7 Receptor-mediated epithelial export of dimeric IgA and pentameric IgM. Membrane secretory component (mSC) functions as polymeric Ig receptor (pIgR) and is expressed basolaterally on serous-type secretory epithelial cells. The polymeric Ig molecules are produced with incorporated J chain (IgA + J and IgM + J) by mucosal plasma cells. The resulting secretory Ig molecules (SIgA and SIgM) mediate first line of defense in the mucus layer on the epithelial surface by performing immune exclusion of antigens (Ag). Although J chain is often produced by IgG-producing mucosal plasma cells (70–90%), it does not combine with IgG (nor with IgD or IgE; not shown) but is degraded intracellularly as indicated ($\pm$ J). Locally produced and serum-derived IgG and monomeric IgA can be transmitted paracellularly to the lumen in amounts depending on the integrity of the epithelial barrier (dashed arrows). Free SC (depicted in mucus) is generated when unoccupied pIgR (top symbol) is cleaved at the apical face of the epithelial cell in the same manner as bound SC incorporated into SIgA and SIgM.

and IgM⁺ PCs often remain predominant up to one month. Thereafter, a rapid expansion of IgA⁺ PCs takes place, and some increase may be seen up to one year of age. Accordingly, only traces of SIgA and SIgM occur in intestinal fluid during the first postnatal period, whereas some IgG is often present – mainly reflecting passive transmission from the lamina propria which, after 34 weeks of gestation, contains readily detectable maternal IgG.²⁹

A much faster establishment of SIgA immunity can be seen in developing countries with a heavy microbial load.⁷⁴ Some reports suggest that probiotic treatment also enhances the production of IgA, but this was not confirmed by measuring IgA in saliva.⁷⁵ Nevertheless, after a combination of prebiotic and probiotic treatment given perinatally and for the first six months of life, infants that showed an early elevation of fecal IgA had reduced risk of allergies before two years of age.⁷⁶ Also, in a recent study it was shown that early intestinal colonization with *Bifidobacterium* species was associated with significantly elevated levels of SIgA in saliva at 6 months of age.⁷⁷ Prebiotics, especially oligosaccharides such as a mixture of galactans and inulin-type fructans (GOS-FOS), increases the fecal

concentration of bifidobacteria; and early-life exposure to GOS-FOS results in significantly elevated fecal levels of SIgA in healthy infants.⁷⁸

2.3.2 The gut epithelial barrier

Although so-called ‘gut closure’ normally occurs in humans mainly before birth, the mucosal barrier may be inadequate up to two years of age; the mechanisms involved remain poorly defined,⁷⁹ but the development of secretory immunity is most likely a decisive variable. Importantly in this context, pIgR-deficient knock-out mice that lack SIgA and SIgM exhibit aberrant mucosal leakiness⁸⁰ and have increased uptake of food proteins and commensal bacteria.⁸¹ A thick virtually microbe-devoid inner mucus layer has been revealed at the apical epithelial surface of the normal rodent colon;⁸² this protected zone apparently limits direct bacterial contact with host cells in the healthy gut, but it may nevertheless be permeable to MAMPs and antigens derived from the abundant microbiota present in the outer mucus layer – particularly when SIgA (and SIgM) is absent.

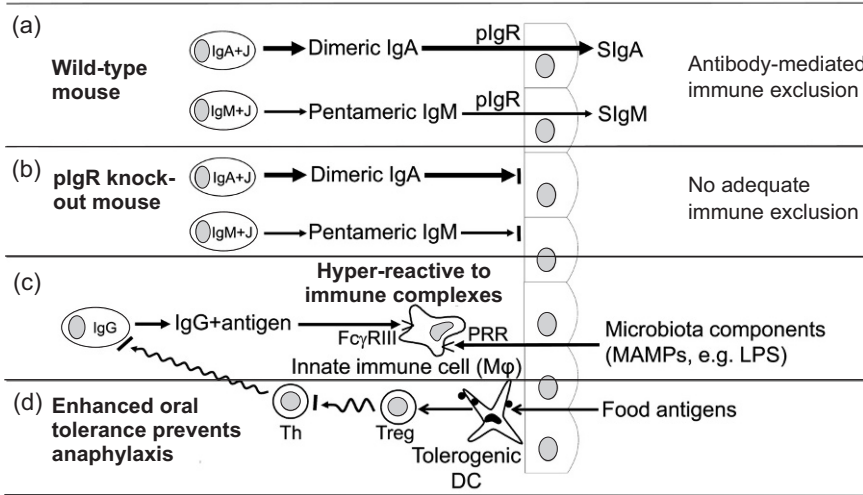


Fig. 2.8 Summary of experimental pIgR-deficient mouse model for hypersensitivity and oral tolerance. (a) The reference situation in pIgR-sufficient mice. (b) Lack of secretory antibodies (SIgA and SIgM) in pIgR knockout mice leads to inadequate immune exclusion of microbiota components derived from commensal gut bacteria. (c) These conserved microbe-associated molecular patterns (MAMPs), e.g. lipopolysaccharide (LPS), will interact with pattern-recognition receptors (PRRs) on innate immune cells, including macrophages (Mφ). The cells therefore become hyper-reactive to IgG-containing immune complexes interacting with Fc receptors (FcγRIII), which renders the mice predisposed to anaphylaxis. (d) The deficient epithelial barrier also allows increased uptake of food antigens from the gut lumen (e.g. fed ovalbumin) which enhances induction of oral tolerance, providing a net anti-inflammatory effect against undue penetration of the same antigen into the body by any route (e.g. dermal). Production of IgG antibodies against this sensitizing antigen will thus be downregulated, and the animal is protected against systemic anaphylaxis after cognate antigen challenge.

Accordingly, the pIgR knockout mice show significantly elevated production of systemic IgG antibodies to commensal bacterial antigens but, interestingly, not to common food proteins such as gluten peptides.^{80,81} The undue influx of microbial products causes a generalized hyper-reactive state with over-activation of the innate cellular (nuclear factor kappa B (NFκB) transcription pathway, resulting in 50% liability of these mice to anaphylactic death after parenteral antigen sensitization with ovalbumin (OVA) and low-dose intradermal challenge with the same antigen.⁸³ However, the pIgR-deficient mice also exhibit enhanced capacity for induction of Treg cells and oral tolerance, which after OVA feeding is fully able to control IgG1- and T cell-dependent hypersensitivity against the antigen (Fig. 2.8).

This observation might imply that at the same time as an inadequate intestinal barrier in the infant represents a risk for hypersensitivity reactions, it will promote tolerance against cognate dietary antigens continuously present in small amounts in the gut.¹⁴ Boirivant *et al.*⁸⁴ likewise reported that a mild or transient breaching of zonula occludens in the intestinal epithelium of mice leads to a dominant anti-inflammatory Treg-cell response. Also of note, children (0.5–4.5 years old) who had grown out of their cow's milk allergy, as revealed by oral challenge after at least two months on a milk-free diet, showed expansion of Treg cells in peripheral blood,^{14,85} probably reflecting that early infancy is the time when oral tolerance is best achieved. The relatively leaky gut epithelium apparently promotes tolerance induced by continuous mucosal exposure to luminal antigens, and the homeostatic balance might be enhanced by cognate SIgA antibodies.

The postnatal balance between the epithelial barrier function and oral tolerance thus seems to be critical for the induction of immunological homeostasis. Notably, although the incidence of food allergy (apparently non-IgE-mediated) is increased in children with IgA deficiency, it is not strikingly elevated⁸⁶ – perhaps because the induction of Treg cells is enhanced in addition to compensatory SIgM, which in such individuals partially replaces the lacking SIgA in the gut.^{35,87} Importantly, bacterial overgrowth occurs in the jejunum of vagotomized patients only when IgA deficiency is combined with suboptimal function of innate defenses such as gastric acid and peristalsis.^{45,88} In this context, it is notable that the frequency of selective IgA deficiency among patients with Crohn's disease in Sweden is significantly increased, that is, one in 100 compared to one in 600 for the healthy population (Lennart Hammarström, personal communication).

AID knockout mice that have SIgA deficiency due to lack of Ig class switching, and also exhibit a defective IgM antibody response due to absent somatic hypermutation (SHM), show massive intestinal overgrowth of commensal anaerobic bacteria with a resulting striking hypertrophy of ILFs over time.⁸⁹ Mice with an AID enzyme that has been mutated in a sequence position allowing CRS but not SHM do have normal intestinal SIgA levels but still show a phenotype similar to AID knockout mice because SIgA as well as SIgM have insufficient bacterial reactivity to regulate gut microbial homeostasis.⁷ The aberrant ILFs that develop have some resemblance to the irregular lymphoid aggregates seen in long-standing IBD.⁹⁰

In contrast to AID and pIgR knockout mice, however, selectively IgA-deficient mice (like IgA-deficient humans) have compensatory SIgM antibodies in their gut lumen.⁹¹ Interestingly, these mice show no increased susceptibility to various gut infections or to dextran sulfate sodium (DSS)-induced colitis, whereas pIgR deficient mice do – probably because they lack both SIgA and SIgM.^{91,92}

It has been suggested that postnatal hyper-reactivity of the immune system may also result from intrauterine events, probably causing genetically or epigenetically determined poor Treg-cell function and immunological immaturity,^{93,94} as also suggested from cord blood studies.⁹⁵ In normal mice it has been shown that neonatal (but not adult) CD4⁺ T cells are strikingly prone to differentiate into Treg cells upon stimulation.⁹⁶ Moreover, the intestinal epithelium itself is equipped with a vast array of features to control immune barrier homeostasis.⁹⁷ These key intrinsic mechanisms have recently been reviewed and include variables such as secretion of mucins and defensins, inflammasome function, intercellular junctional complex regulation and PRR signaling.⁹⁸ As discussed in a subsequent section, the latter is downregulated in the neonatal gut epithelium to preserve its integrity upon the encounter with the commensal microbiota.^{99,100}

2.3.3 Additional IgA functions in gut defense

Antibody production by the numerous intestinal pIgA⁺ PCs may also be important for homeostasis within the lamina propria as a result of several anti-inflammatory mechanisms. IgA lacks ordinary complement-activating properties¹⁰¹ and can therefore block non-specific biological amplification triggered by locally produced or serum-derived IgG anti-bodies, which may actually increase the penetration of exogenous bystander antigens through the surface epithelium.¹⁰² This is important in view of the fact that immune complexes are probably formed even within the normal lamina propria due to some influx of soluble antigens, particularly following food intake.²⁷ Also, *in vitro* and *in vivo* experiments have suggested that soluble antigens – after pIgA-mediated non-inflammatory trapping in immune complexes – may be cleared by the secretory epithelium via pIgR-mediated translocation to the lumen.¹⁰³ Similar experiments have suggested that pIgA antibodies can neutralize lipopolysaccharide (LPS) and viruses within secretory epithelial cells during pIgR-mediated SIgA export,^{4,5} and thus return harmful microbial components to the gut lumen (Fig. 2.9). Mouse models have confirmed that the latter mechanism contributes to the intestinal defense against rotavirus infection,¹⁰⁴ and IgA⁺ PCs with specificity for rotavirus occur in normal jejunal mucosa of adults with no signs of current infection.¹⁰⁵

The mucosal clearance function exhibited by pIgA (and probably pentameric IgM) antibodies reinforces the immune exclusion mediated by SIgA (and SIgM) with innate-like low-affinity antibody activity generated against commensal bacteria, and which to some extent may be cross-reactive.^{8,45,106} The high-affinity antibodies induced by pathogens or properly adjuvanted oral vaccines may contribute even more efficiently to both immune exclusion and mucosal clearance, as reviewed elsewhere.⁵

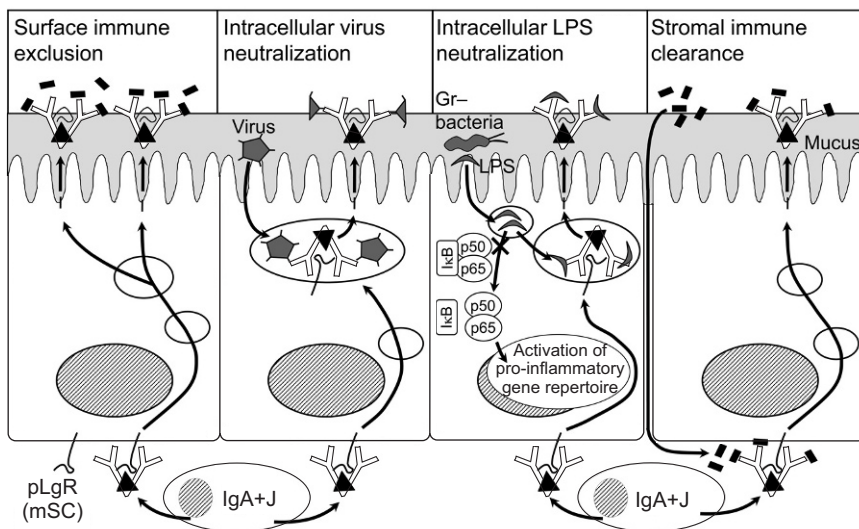


Fig. 2.9 Three levels at which dimeric IgA and secretory IgA (SIgA) antibodies may provide mucosal defense after being produced with J chain (IgA + J) by plasma cells in the gut lamina propria. Left panel: dimeric IgA is transcytosed by the polymeric Ig receptor (pIgR), also known as membrane secretory component (mSC), across epithelial cells and released from apical endosomes to the lumen as SIgA that performs immune exclusion by interaction with antigens (short bars). Middle panels: dimeric IgA antibodies interact with viral antigens or lipopolysaccharide (LPS) from Gram-negative (Gr-) bacteria within apical endosomes during pIgR-mediated transcytosis, thereby performing intracellular virus or endotoxin neutralization and clearance, which inhibits induction of inflammation and epithelial damage. Panels schematically depict the way in which LPS is diverted from activating the pro-inflammatory NFκB pathway in the epithelial cell, thereby inhibiting secretion of, for instance, pro-inflammatory IL-8. Right panel: dimeric IgA antibodies interact with penetrating antigens in the lamina propria and shuttle them back to the lumen by pIgR-mediated export.

Locally produced pIgA may further influence homeostasis by interacting with the Fcα receptor (FcαRI, CD89) on leukocytes in the lamina propria. First, on the one hand it has been shown that pIgA-containing immune complexes are able to suppress attraction of neutrophils, eosinophils and monocytes, thereby reducing their pro-inflammatory activities.¹⁰⁷ On the other hand, when such complexes interact with CD89 on neutrophils, leukotriene B4 is released as a chemotactic factor attracting more of these cells.¹⁰⁸ Second, IgA can apparently downregulate the secretion of pro-inflammatory cytokines such as TNF-α from activated monocytes.¹⁰⁹ However, it is uncertain whether this mechanism operates in the normal gut because mucosal macrophages do not express detectable surface CD89 – at least not in the small intestine.^{110,111} Third, neutrophil and monocyte activation that results in generation of reactive oxygen metabolites ('respiratory burst') is reportedly inhibited by IgA.¹¹² Conversely, pIgA may temporarily trigger monocytes to enhanced activity – including TNF-α secretion¹¹³ – and IgA (particularly SIgA)

appears to be a potent activator of eosinophils.^{114,115} Indeed, complexed SIgA induces respiratory burst and degranulation of these cells, while soluble SIgA enhances their survival *in vitro*.¹¹⁶

Together, these *in vitro* results suggest that the participation of pIgA in mucosal homeostasis is quite fine-tuned¹¹⁷ – perhaps being skewed towards a pro-inflammatory potential in IBD where there are numerous granulocytes¹⁰⁸ along with recently recruited monocyte-like macrophages which express the LPS co-receptor CD14 and PRRs such as TLR2 and TLR4.^{118,119}

Initially, the shift from the normal predominance of mucosal pIgA production to IgG and monomeric IgA in IBD lesions may represent a powerful second line of defense because these antibodies may efficiently mediate immune elimination of penetrating bacteria via phagocytosis and antibody-dependent cell-mediated cytotoxicity.^{107,120} Notably, by means of CD89 both neutrophils¹⁰⁸ and liver Kupffer cells¹²¹ may phagocytose translocated gut bacteria opsonized even with serum-type monomeric IgA. Rodent studies suggest that the liver is designed to handle this antigen elimination in a silent manner because of its unique tolerogenic capacity, including induction of Treg cells by plasmacytoid DCs.¹²² Antigen elimination within the intestinal lamina propria, however, may more readily cause inflammation and tissue damage when productive systemic type of immunity is involved;^{107,120} this is a risk the immune system sometimes must take to hinder sepsis and save life. The role of mucosal IgA and oral tolerance is to counteract over-activation of such potentially harmful immune reactions.

2.4 Immunological consequences of gut microbial–host interactions

2.4.1 Effect on epithelium and secretory immunity

In fetal life, murine gut epithelial cells are sensitive to microbial factors such as LPS (endotoxin) because they express intracellularly a PRR for this MAMP, namely TLR4.⁹⁹ Exposure to LPS in the vaginal tract during birth activates the neonatal gut epithelium via TLR4 and temporarily upregulates microRNA-146a, which leads to degradation of the TLR signaling molecule IL-1R-associated kinase 1 (IRAK1). Such translational repression of IRAK1 protects the epithelium from microbiota-induced damage during transition from a principally sterile environment.^{100,123} In remarkable contrast, epithelial tolerance to the commensal bacteria does not occur in mice delivered by cesarean section.⁹⁹ These experimental observations may be related to the fact that children delivered by cesarean section appear particularly prone to develop food allergy, especially if they have a genetic predisposition for atopy.^{124,125}

Mouse experiments have also demonstrated a crucial postnatal role of commensal gut colonization both in establishing and regulating the epithelial barrier, including upregulation of pIgR expression.^{126,127} Such beneficial effects of microbiota-derived MAMPs appear again to be mediated largely via PRRs, particularly TLRs

and similar innate sensors on the epithelial plasma membrane (apically or basolaterally) or on endosomal membranes.^{97,128} Cell culture experiments with the polarized human colon carcinoma cell line HT-29 have shown that ligation of TLR3 and TLR4 with the respective MAMPs (double-stranded RNA and LPS) from the apical side upregulates pIgR expression,¹²⁹ which may then increase the pIg export and thus enhance secretory immunity.^{45,130} Gut bacteria of the family *Enterobacteriaceae* can induce this effect.¹³¹

2.4.2 The importance of both diet and microbiota

Several cytokines derived from activated Th cells or APCs may likewise upregulate pIgR expression and thereby increase the export of secretory antibodies in response to microbial stimulation.^{45,56,130} A similar enhancing effect has been shown for butyrate in combination with various cytokines.^{45,132} Butyrate is one of the short-chain fatty acids (SCFAs) derived from anaerobic microbial fermentation of complex plant polysaccharides in the gut; it acts as an important energy source for colonic epithelial cells and is thus in several ways involved in maintaining the intestinal barrier.¹³³ Notably, SCFAs can increase or dampen gene transcription levels of specific DNA sequences by signaling through G protein-coupled receptors. Thus, butyrate can contribute to the anti-inflammatory tone of the healthy gut by inhibiting activation of the transcription factor NFκB.¹³³ Both epidemiological studies and animal experiments indicate that a diet rich in plant fibers protects against immune-mediated inflammatory disease; but it is unclear to what extent this effect can be ascribed to SCFAs or to an altered composition of the gut microbiota.¹³³

Vitamin A is also an important dietary factor supporting secretory immunity, not only through the positive effect of RA on cytokine-induced pIgR expression in intestinal epithelial cell at the transcriptional level,^{134,135} but also by enhancing the IgA switch and intestinal homing of mucosal B cells, as previously discussed. Nevertheless, it has been reported that under-nourished children respond to bacterial overgrowth in the gut with enhanced intestinal production and external transport of IgA.¹³⁶ Mouse experiments have suggested that when RA is present in such a pro-inflammatory environment, it may paradoxically downregulate oral tolerance and boost inflammation, thereby jeopardizing the gut barrier rather than preserving it.¹³⁷ Importantly, the detrimental effects of severe malnutrition on the SIgA system seem to be reversed with nutritional rehabilitation.¹³⁸

The separate impact of microbial and dietary antigens on intestinal immunity was clearly shown in a study based on whole gut lavage obtained from healthy adult volunteers;¹³⁹ in Dhaka (Bangladesh) the intestinal total IgA concentration was almost 50% higher than that of comparable samples collected in Edinburgh (UK), and the IgA antibody titer against LPS core types of *E. coli* was almost seven times higher in the former group of subjects, in contrast to the lower levels of OVA antibodies.

Altogether, there are many mechanisms by which exogenous variables may enhance intestinal IgA production and pIgR expression above the constitutive

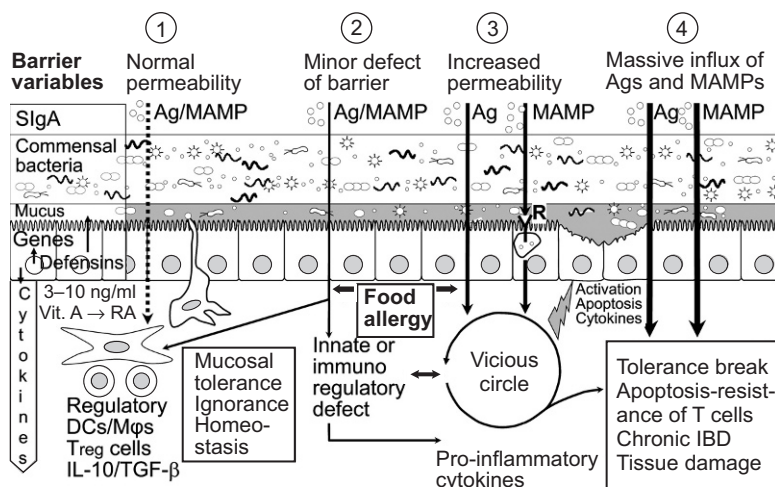


Fig. 2.10 Model for maintenance of mucosal homeostasis in the gut and abrogation of oral tolerance. Epithelial barrier variables, including secretory IgA (SIgA), mucus, defensins, genes (e.g. regulating tight junctions), and cytokines are indicated in the panels to the left. 1. Normal epithelial permeability allows some uptake of MAMPs (microbe-associated molecular patterns) and food antigens (Ags), with amounts of dietary proteins detected in the circulation after a meal indicated. 2. Minor barrier defects result in increased uptake, but mucosal homeostasis is maintained when oral tolerance is adequately induced by quiescent dendritic cells (DCs) and macrophages (Mφs), which can convert dietary vitamin A to retinoic acid (RA) to aid in their induction of regulatory T (Treg) cells providing the suppressive cytokines IL-10 and TGF-β. If there is a regulatory defect, a vicious circle develops which reciprocally acts on the immunoregulatory network. 3. With more skewing towards regulatory dysfunction, the vicious circle will activate the epithelium, and the MAMP and Ag uptake is enhanced (as suggested by the graded arrows), both by increased permeability and aberrant receptor (R) expression apically on epithelial cells. Food allergy probably develops in the dysfunctional zone between '2' and '3', as indicated. 4. The adverse development may finally reach a 'point of no return' and result in epithelial activation with apoptosis and increased secretion of pro-inflammatory cytokines, followed by chronic inflammation with apoptosis-resistant effector T cells as seen in inflammatory bowel disease (IBD).

level and thus reinforce immune exclusion. In addition, polarized gut epithelial cells seem to retain their ability to dampen the pro-inflammatory effect of PRR-mediated microbial signals coming from the luminal side,^{127,128,140} but after bacterial invasion, PRR signaling from the basolateral side results in NFκB activation with release of epithelial defensins to join the combat against infection.^{97,141} Accumulating evidence suggests that barrier-related homeostasis depends on 'cross-talk' between the epithelium (via cytokines and other factors) and lamina propria cells including macrophages, DCs and T cells.¹⁴²⁻¹⁴⁵

Thus, when immune regulation is operating in a healthy manner, the small amounts of MAMPs and exogenous antigens from microbes and food penetrating into the lamina propria are handled in a homeostatic manner by DCs and macrophages, with a balanced cytokine secretion and induction of Treg cells (Fig. 2.10)

However, if the influx is excessive or regulatory mechanisms are defective, immune reactions may be driven into hypersensitivity and enter a vicious circle with pro-inflammatory cytokines and epithelial apoptosis.^{14,144–146} In this manner, a 'point of no return' may be reached, as seen in IBD with apoptosis-resistant pathogenetic T cells and tissue damage (Fig. 2.10). The take-home lesson from most disease models in gene-manipulated animals is that a predilection exists for immunopathology to occur in the distal gut – where most commensals reside¹² – when adaptive immunity is dysregulated and innate immunity or the intestinal barrier function compromised.^{147,148}

2.4.3 Homeostasis tailored by SIgA interacting with commensals

A great proportion of the commensal bacteria in the healthy human gut or at other mucosal surfaces are coated with IgA,^{149,150} most likely representing low-affinity SIgA antibodies.^{23,61} Such microbial–host interactions contain the commensal microbiota without eliminating it. Thus, it has been shown in a murine infection model with *Salmonella typhimurium* that bacteria coated with innate-like, cross-reactive SIgA antibodies showed reduced shedding and less liability to horizontal spreading by the fecal–oral route.^{91,151}

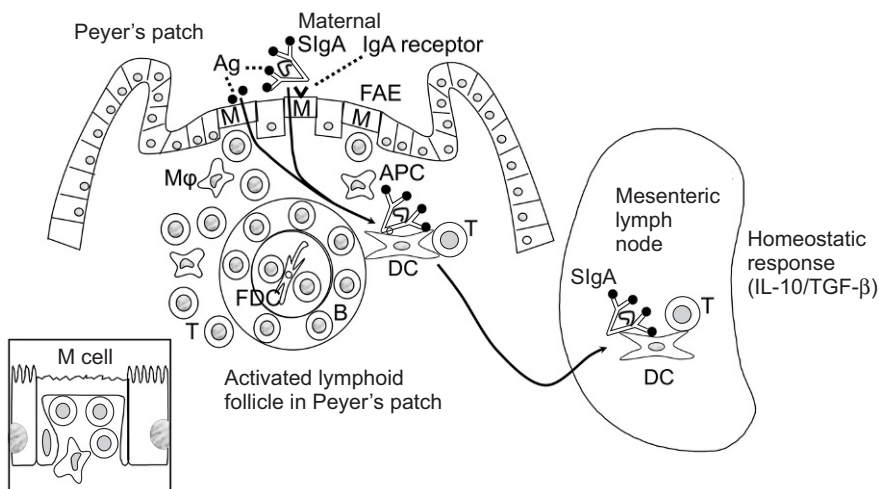


Fig. 2.11 Integration of the immune systems of the mother and her breastfed baby via targeted M cell-mediated antigen (Ag) uptake. Secretory IgA (SIgA) antibodies in breastmilk may guide induction of the infant's intestinal immune system because M cells in the follicle-associated epithelium (FAE) of Peyer's patches express an as yet uncharacterized receptor for IgA. This receptor may facilitate uptake of antigens that have formed immune complexes with cognate maternal SIgA antibodies in the gut lumen of the infant. The complexes may be further targeted to sub-epithelial antigen-presenting cells (APCs), such as macrophages (Mφ) and dendritic cells (DCs), which carry them to mesenteric lymph nodes where a homeostatic immune response dominated by secretion of IL-10 and TGF-β is induced. Based on experimental data provided by Corthésy.⁴ An M-cell pocket with its cellular content is schematically shown in the panel to the left.

Mouse experiments have further documented that a gut IgA response to a single commensal microbial epitope (capsular polysaccharide A, PSA) can be immunomodulatory and protect against pathogen-induced colitis.³² Concurrently, PSA from the prominent commensal *Bacteroides fragilis* induces potent Treg cells.¹⁶² Importantly, germ-free mice monocontaminated with the commensal *Bacteroides thetaiotaomicron* demonstrate that specific SIgA antibodies directed against PSA inhibit activation of innate response markers such as oxidative burst and NFκB, thereby inducing crucial modulation of immune homeostasis in the gut as well as microbial antigenic drift.¹⁵³ SIgA antibodies can hence control the intestinal microbiota in a non-inflammatory, symbiotic or mutualistic relationship with the host.¹⁵⁴

Experimental studies have also revealed how intestinal homeostasis is mediated by microbial–host interactions in mice monocolonized with the *Clostridia*-related segmented filamentous bacterium (SFB) which particularly grows in the distal ileum.^{155,156} SFB adheres to murine Peyer's patches and stimulate T-cell as well as IgA responses. Here a distinction is needed between B1 (T cell-independent) and B2 (T cell-dependent) responses when comparing Peyer's patches and lamina propria; this distinction is as yet not clear in mice.^{8,50} It is also possible that GALT may take up immune complexes containing SIgA bound to commensals via as yet uncharacterized receptors for IgA on M cells and then induce a homeostatic response (Fig. 2.11). Recently this mechanism was suggested to direct the opportunistic bacterium *Alcaligenes* into murine GALT and thereby make these tissue structures more resistant to pathogen invasion.¹⁵⁷

2.5 IgA and breastfeeding in immune control

The level of SIgA appears to contribute to an individual's threshold for hypersensitivity reactions to exogenous antigens.¹⁴ Thus, the risk of contracting allergy seems increased when the development of the IgA system is retarded and the SIgA-dependent barrier function insufficient.¹⁵⁸ Minor dysregulations of both innate and adaptive immunity (especially IgA) have been observed in children with multiple food allergy.¹⁵⁹ These clinical observations are in accordance with the hyper-reactivity (Fig. 2.8) and sensitivity to DSS-induced colitis⁹² seen in pIgR knockout mice.

It is therefore not surprising that exclusive breastfeeding up to the age of at least four months has an allergy-preventive effect, also in families without atopic heredity.^{160–162} It has recently been argued that prolonging this period increases the risk of food allergy and celiac disease.¹⁶³ However, taking into account the infection-preventive effect of breastfeeding, the consensus in the industrialized part of the world seems to favor introducing solid food alongside continued mother's milk at four to six months.¹⁴ In developing countries breastfeeding should be continued even longer than the one year recommended in Westernized societies.

In addition to the remarkable reinforcement of mucosal defense provided by maternal SIgA (and SIgM) antibodies as a natural immunological 'substitution

therapy', it is important to emphasize the positive nutritional effect of breastfeeding on immune development.¹⁵⁸ Mother's milk also contains a number of immune cells, cytokines and growth factors that may exert a significant biological effect in the breast-fed infant's gut, apparently enhancing in an indirect way even the subsequent health of the individual.^{40,158,164} Moreover, its high content of oligosaccharides serves as a prebiotic promoting the growth of lactic acid-producing bacteria and reducing anaerobic bacteria in the gut of breast-fed infants.¹⁵⁸ Probiotic bacteria can reportedly also occur in breastmilk,¹⁶⁵ perhaps after being transported to the mammary glands from the gut by DCs.¹⁶⁶ Having been breast-fed for at least three months protects against both Crohn's disease and ulcerative colitis in adulthood,¹⁶⁷ and probably also against pediatric IBD.¹⁶⁸ Importantly, mothers with IBD receiving anti-inflammatory drugs can in most cases safely breastfeed their babies, and it may even provide a protective effect against disease flare.^{169–171}

Numerous studies on the effect of breastfeeding on the development of secretory immunity have been performed with salivary IgA measurements as a read-out system. Discrepant observations have been made and the influence of contaminating the sample with milk SIgA, shielding of the breast-fed infant's mucosal immune system by maternal SIgA antibodies, and altered growth and composition of the infant's gut microbiota have been discussed as possible uncontrollable variables. Moreover, several prospective studies have reported that the postnatal increase of salivary IgA (and IgM) initially is more prominent in formula-fed than in solely breast-fed infants.²⁹

Nevertheless, evidence suggests that breastfeeding promotes the development of secretory immunity over time,^{30,158} apparently even at extra-intestinal sites such as the urinary tract.¹⁶⁴ Thus, although breastfeeding initially may reduce the induction of SIgA, it appears later on in infancy (up to eight months) to boost secretory immunity. As mentioned above, one possibility is that SIgA antibodies in mother's milk guide the uptake of cognate luminal antigens via receptors for IgA on M cells (Fig. 2.11); as suggested by mouse experiments, the antigens may further be targeted to DCs which migrate to MLNs where they induce a homeostatic immune response.⁴ Therefore, the remarkable output of SIgA during feeding represents an optimally targeted passive immunization of the breast-fed infant's gut,⁴⁰ and might serve as a positive homeostatic feed-back loop.

Traces of exogenous antigen transferred into the milk of mothers may thus contribute to the induction of oral tolerance in the breast-fed infant; therefore, allergen avoidance during lactation is generally no longer recommended.¹⁴ Various mouse models have indeed documented that antigen appears in the milk of exposed mothers and that it can induce Treg cells in the suckling neonate, either together with TGF- β or after complexing with IgG antibody and transfer by FcRn into gut mucosa.^{172,173} In human milk, maternal SIgA-containing immune complexes may perform a similar function (Fig. 2.11). Interestingly in this context, a recent systematic review of published studies on the TGF- β levels in human milk suggested that this cytokine protects against allergy in breast-fed infants and young children.¹⁷⁴ It was also recently reported that genes that are modulated during epithelial gut differentiation in the neonate are differentially expressed in breast-

fed and formula-fed infants.¹⁷⁵ It is unknown whether this was a direct effect of milk components or caused by differences in the microbiota of the two feeding groups.

2.6 Induction of homeostasis *versus* hypersensitivity in the gut

2.6.1 Oral tolerance in humans

It is believed that oral tolerance is largely explained by different T-cell events such as anergy, clonal deletion and induction of Treg cells by conditioned APCs (Fig. 2.12). However, other regulatory principles may clearly be involved.^{10,14,30,158,176–179} For ethical reasons, the existence of mucosally induced tolerance in human beings

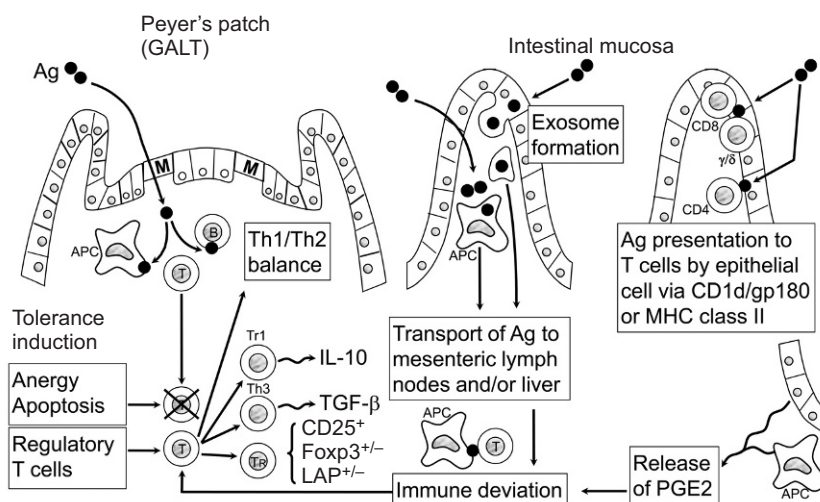


Fig. 2.12 Putative mechanisms of oral tolerance induction. Hyporesponsiveness to innocuous antigen (Ag) that gains access to immune cells through M cells of gut-associated lymphoid tissue (GALT), or through the mucosal surface epithelium, may be explained by T-cell anergy, clonal deletion by apoptosis, and active (contact-dependent) or cytokine-mediated (immune deviation) suppression exerted by various subsets of regulatory T (Treg) cells. Induction (or expansion) of Treg cells takes place locally or at distant sites such as the regional lymph nodes or the liver after the dissemination of soluble Ag or the transport of Ag by antigen-presenting cells (APCs) or epithelial exosomes. In this manner, mucosal and peripheral hyperactivation of effector T cells is avoided. CD25⁺ Treg cells are either positive or negative for the transcription factor Foxp3 and/or latency-associated peptide (LAP^{+/-}), and certain subsets (Tr1, Th3) produce the suppressive cytokines IL-10 and TGF- β . Treg cells are important for the development of a balanced Th1/Th2 cytokine profile. The suppressive tone of the gut may also be driven by unconventional Ag presentation by epithelial cells to intraepithelial or subepithelial T cells of various phenotypes (CD8⁺, CD4⁺, γ/δ ⁺), and the immune deviation effect of prostaglandin E2 (PGE2) released from APCs or the epithelium.

is supported mainly by circumstantial evidence. Thus, the gut mucosa of healthy individuals contains virtually no hyperactivated T cells and hardly any pro-inflammatory IgG production, and their serum levels of IgG antibodies to food antigens are low.¹⁵⁸

Moreover, the systemic IgG response to dietary antigens tends to decrease with increasing age,^{180,181} and a hyporesponsive state to bovine serum albumin has been demonstrated by intradermal testing in adults.¹⁸² Also, as mentioned previously, there is evidence from several studies that children who grow out of their food allergy develop or re-establish oral tolerance with induction or expansion of Treg cells.^{14,85} The promising clinical results obtained by treating allergic patients with the allergen as a 'tolerogen' can likewise be explained by oral tolerance induction.¹⁴

Interestingly, nasal application or feeding of a novel antigen (keyhole limpet hemocyanin) in healthy people induced peripheral downregulation of T-cell immunity and, less consistently, also suppressed systemic antibody responses to subsequent parenteral immunization.^{183,184} By contrast, oral tolerance could not be induced in patients with IBD where the epithelial barrier is severely deteriorated and the immunogenetics favors mucosal inflammation.^{185,186}

2.6.2 Intestinal tolerance induction

In healthy human gut mucosa, resident APCs are quite inert in terms of immune-productive stimulatory properties,¹⁸⁷ and they hardly express detectable surface levels of TLR2 or TLR4.¹¹⁹ Also, only negligible expression of the LPS co-receptor CD14 is normally observed on these cells, and their pro-inflammatory cytokine response is usually low after LPS stimulation.^{110,188} Nevertheless, the phagocytic and bacteriocidal activity of mucosal macrophages is maintained,¹⁸⁹ which would be important for silent clearance of commensal bacteria normally penetrating into the mucosa in small numbers.^{13,23,190}

These observations support the notion that both macrophages and DCs play a central role in oral tolerance,¹⁴² and most human intestinal APCs come from a common myeloid progenitor and often show an intermediate phenotype.^{191,192} Heterogeneity of murine lamina propria APCs has also been highlighted.^{193,194} Recent *in vivo* observations in mice suggest that DC development from monocytes to the phenotype DC-SIGN/CD209⁺ is a predominant pathway when abundant LPS is available,¹⁹⁵ such as in the gut. The subepithelial band of monocyte-derived CD103-CX3CR1⁺ DCs in the murine gut mucosa is indeed macrophage-like¹⁹² and a source of IL-10 which contributes to expansion of Treg cells.¹⁹⁶

In a quiescent steady state, however, mucosal CD103⁺CCR7⁺ DCs (and possibly macrophages) are migratory and carry penetrating dietary and innocuous microbial antigens away from the gut mucosa to the MLNs.^{39,197–199} Here a portion of the same cells, in a maturation process, become further conditioned for tolerance induction and drive the expansion of Treg cells.^{30,200} It has been shown in humans that the tolerogenic properties of these migrating mucosal DCs partly depend on expression of indoleamine 2,3-dioxygenase (IDO)²⁰¹ – an enzyme known to be

involved in DC induction and function.²⁰² Hyperactivation of effector T cells in the intestinal mucosa with accompanying inflammation can thus be inhibited by this DC-dependent regulatory mechanism, both initially and subsequently. This is so because homeostatic control is also exerted when the generated Treg cells home from MLNs to the lamina propria where they expand to install oral tolerance.¹⁴ Such homing of Treg cells to the gut mucosa seems to be particularly active in infancy.²⁰³

In the healthy gut a dietary effect on the induction of Treg cells is exerted through the conversion of vitamin A to RA by the enzyme RALDH, which is expressed both by intestinal DCs, macrophages and epithelial cells,^{41,193} as well as by MLN stromal cells.²⁰⁴ Together with IL-2, TGF- β and IL-10, RA can drive the induction of Treg cells.^{205–208} Mouse experiments have suggested that the retinol-metabolizing activity of small intestinal CD103⁺ DC is in itself imprinted locally by vitamin A or bile retinoids.²⁰⁹ As alluded to previously, however, in pro-inflammatory conditions RA may act as a co-adjuvant to cytokines such as IL-15 rather than maintaining gut homeostasis.¹³⁷

In human peripheral tissues Treg cells are heterogeneous and can apparently differentiate either by conversion from a naïve (CD45RA⁺) phenotype or from rapidly proliferating T cells with a memory/effector (CD45RO⁺) phenotype with different migratory properties.^{210,211} It is the latter that expresses high levels of the transcription factor Foxp3 and appears actively suppressive.²¹² In the presence of IL-2, vitamin D also seems to stimulate the expression of Foxp3 as well as another potential effector molecule of human Treg cells, namely CTLA-4.²¹³

Altogether, human Treg cells are functionally and phenotypically more diverse than the murine counterparts, including the expression of the activation markers CD25, CD127 and inducible co-stimulatory (ICOS) molecule, as well as the Foxp3 transcription factor.^{214–216} There is also a need for better understanding of the functional stability and plasticity (e.g. acquisition of a Th17 phenotype) of Treg cells.²¹⁷

2.6.3 Sensing of microbial components

Final cellular conditioning for oral tolerance in MLNs appears to depend on appropriate stimulation of the migrating mucosal APCs by certain MAMPs derived from commensal bacteria (Figs 2.12 and 2.13), which induce the signaling molecules and transcription factors dictating the differentiation pathways and cytokine profiles of the activated T cells.^{13,14,218} Components of intestinal parasites such as helminths can exert similar immune modulation^{12,17,219–221} – apparently to some extent through induction of Treg cells by mimicking the effect of TGF- β by ligation of its receptor.²²² Indeed, helminth-derived immunomodulators may be important in future novel medication for immune-mediated pathologies such as IBD.²²³ Also of note, several studies suggest that LPS and other factors present in dust on animal farms play a central role in the early programming of the immune system.^{224–226}

There is also an ongoing search to find out if allergy to food and aeroallergens is associated with hereditary single nucleotide mutations (polymorphisms) in

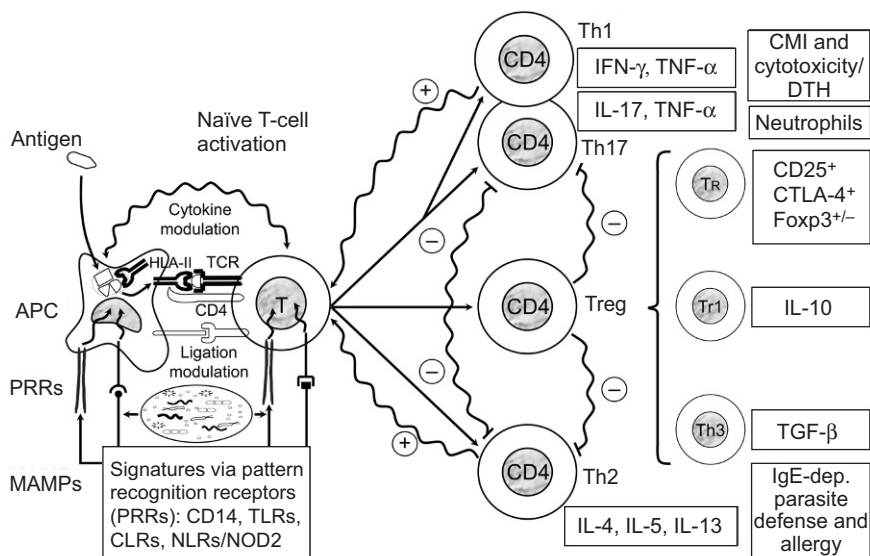


Fig. 2.13 Decision-making in the mucosal immune system is modulated by co-stimulatory signals (cytokines and ligands) operating in the synapse between antigen-presenting cell (APC) and T cells. Activation of naïve CD4⁺ T cells occurs when the APC takes up antigen and processes (degrades) it to immunogenic peptides for display to the T-cell receptor (TCR) in the polymorphic groove of HLA class II molecules (HLA-II). The level of co-stimulatory signals determines T-cell modulation (activation and conditioning). When CD4⁺ Th cells are primed for productive immunity, they differentiate into Th1, Th17 or Th2 effector cells with polarized cytokine secretion as indicated. Such skewing of the adaptive immune response depends on transcription factors induced by signals from microbial components and the resulting microenvironmental factors, including cytokines. Bacterial endotoxin (lipopolysaccharide), lipoproteins, unmethylated CpG DNA and other conserved structural motifs are called microbe-associated molecular patterns (MAMPs); they are sensed by cellular pattern-recognition receptors (PRRs) such as CD14, Toll-like receptors (TLRs), C-lectin receptors (CLRs) and NOD-like receptors (NLRs), including NOD2. Signaling from PRRs to the nucleus of APCs and T cells induces various degrees of activation and functional maturation of APCs and will thereby via transcription factors, dictate differential expression of co-stimulatory molecules and cytokines. The cytokines induce various adaptive defense mechanisms or immunopathology (right panels) and exhibit positive and inhibitory feedback loops. Various subsets of regulatory T (Treg) cells can further suppress Th1, Th17 and Th2 effector cell responses. CTLA-4, cytotoxic T-lymphocyte antigen 4; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor. CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity.

PRRs recognizing LPS and other MAMPs, such as CD14, TLR2, TLR4 and NOD.^{227,228} The extended hygiene hypothesis implies that suboptimal PRR stimulation, with delayed maturation of the mucosal immune system and insufficient induction of Treg cells, contributes significantly to the increasing incidence not only of allergy – commonly reflecting over-activation of Th2 cells – but also of other immune-mediated inflammatory disorders – reflecting over-activation of

Th1 or Th17 cells, both of which are normally important for proper defense against infections.^{12,17} Likewise, in the perspective of evolution, Th2 cells have had a crucial role in host defense against parasites.²²⁹

This basis for the hygiene hypothesis has been tested in several clinical studies evaluating the beneficial effect on immune homeostasis exerted by probiotic microbial preparations derived from the commensal gut bacteria and eggs of the porcine helminth (whipworm) *Trichuris suis*.^{17,221} In this context, viable strains of lactobacilli and bifidobacteria have been reported to enhance IgA, both in humans and experimental animals, but these responses have not translated convincingly into clinical effects.^{230,231} To select the right probiotic bacterial strains, or symbiotic combinations with prebiotics or breastmilk, remains a difficult task – and there are safety issues.^{232,233} Experimental studies have indicated that certain strains may in fact be pro-inflammatory while others may induce DCs, directly or via epithelial mediators, to exert anti-inflammatory effects.²³⁴

It is unknown whether probiotics and prebiotics might work mainly through SIgA-mediated reinforcement of the barrier function, expansion of Treg cells or the involvement of both these anti-inflammatory mechanisms – perhaps combined with direct strengthening of epithelial integrity (Fig. 2.10). Notably, the most promising results have been reported for atopic eczema.²³⁰ This skin disease is often seen in patients with IgE-mediated food allergy (20–40%) and is particularly associated with loss-of-function mutations in the filaggrin gene, which is involved in the epidermal barrier function.²³⁵ Similar mutations appear to predispose for the combination of atopic eczema and asthma.²³⁶ These findings apparently reflect that a leaky surface epithelium anywhere in the body may be a predisposing condition for allergen penetration, and that food allergy could be a consequence rather than a cause of atopic eczema.¹⁴ Therefore, the use of this disorder as a clinical read-out of intervention effects on food allergy may not be scientifically acceptable.

2.6.4 Innate signals dictating homeostasis

Micro-organisms have inhabited Earth for at least 2.5 billion years, and the power of the immune system is a result of co-evolution in which commensal bacteria in particular have shaped host defense in a state of mutualism.^{12,17} The prevailing mucosal homeostasis in the gut mucosa is indeed remarkable because of the large surface area to be defended – continuously being exposed to a huge number of different bacterial species as the gut microbiome is perhaps 100 times greater than the human genome.²³⁷ In addition, the human gut harbors an unknown number of viruses.²³⁸ Thus, while the intestine represents the largest exposure to micro-organisms, it is also a major route of exposure to exogenous protein antigens (i.e., food) and micronutrients with immunomodulatory properties.¹⁴

The original hygiene hypothesis postulated that the increasing incidence of allergy in westernized societies was explained by reduced or aberrant microbial exposure early in infancy, resulting in too little Th1-cell activity and therefore an insufficient IFN- γ level to downregulate optimally the prenatal Th2-cell responses which apparently can be ascribed to the cytokine thymic stromal lymphopoietin

(TSLP) secreted by placental trophoblasts.²³⁹ In this context, an appropriate postnatal encounter with a balanced commensal microbiota and exposure to foodborne and orofecal pathogens could probably exert an important homeostatic impact,^{240,241} both by enhancing the SIgA-mediated barrier and by promoting oral tolerance through a shift from the predominant Th2-cell activity in the newborn period.²⁴² The extended hygiene hypothesis postulates that induction of Treg cells is an important part of such microbe-driven homeostasis to avoid both allergy and other immune-mediated inflammatory disorders such as IBD.¹⁷

Naturally occurring Treg cells with suppressive properties are present in large numbers in human fetal MLNs,²⁴³ probably as part of a peripheral tolerance to keep autoreactive effector T cells in check to avoid inflammation and tissue damage.²⁰⁸ These Treg cells are apparently induced in the thymus and expanded in the periphery.^{244,245} After birth, the decision for induction of hyporesponsiveness against innocuous exogenous antigen, *versus* potentially harmful systemic-type productive immunity, may be largely instructed in mucosa-draining lymph nodes such as MLNs. As discussed above, the driving force in this homeostatic mechanism appears to be the microbial impact that conditions APCs and T cells for tolerance by balancing polarizing cytokines induced via PRRs (Fig. 2.13).

Thus, MAMPs do not only directly modulate the epithelial barrier function of neonates^{99,100} but also the activation profiles of innate and adaptive immune cells (Fig. 2.10). Appropriate balancing of the immune system appears to depend on a fine-tuned ‘cross-talk’ between APCs/innate immunity and T cells/adaptive immunity during certain windows of opportunity, particularly early in the newborn period,^{14,17,30} and probably even late in fetal life.²⁴⁶ Concepts such as epigenetic programming *in utero* and subsequent epigenetic regulation are being considered as critical pathways through which environmental changes could alter expression of genes which lead to immune homeostasis or dysregulation,⁹⁴ apparently also involving Treg cells and the Th1/Th17:Th2 balance.^{247,248} It is possible that opportunities for reprogramming may be a lifelong process, thus explaining the late emergence of immune-mediated diseases in some individuals.

2.6.5 Effects of genes *versus* the environment

The increasing incidence of immune-mediated diseases in affluent societies indicates that susceptibility genes for dysregulation are quite universal, such that they can be induced readily with environmental change. Epigenetics is an active research field providing novel understanding of how the environment can have heritable genomic effects and promote disease.^{94,228,248} A number of early life exposures, including dietary nutrients and microbial exposure *in utero*, have recently been shown to have effects on gene expression with an impact on the clinical phenotype (Fig. 2.1). For instance, mice born to dams exposed to bacteria during pregnancy experienced less allergy than those born to unexposed mothers, and maternal TLR signaling was needed for this transmission of protection.²⁴⁹ Even the sensitivity of gut epithelial cells to LPS exposure via TLR4 may be subjected to epigenetic regulation.²⁵⁰

However, the heredity of polygenic diseases is complex and the family history remains the best prediction of both allergy²⁵¹ and IBD.²⁵² Hopefully, the apparently inherent plasticity of the immune system may in the future provide opportunities for reprogramming to facilitate more effective prevention and treatment of these disorders. Thus, even APCs of adults can be conditioned to induce Treg cells by environmental factors such as LPS and cell wall lipids from parasites.^{253,254} Moreover, transient infestation with porcine helminths has been shown to have a beneficial effect on mucosal homeostasis in adult IBD patients,²²¹ and the same has been shown in experimental models of allergy.²²⁰

2.6.6 The importance of commensal bacteria and diet

Indigenous gut bacteria play a central role in the extended hygiene hypothesis. Thus, the intestinal microbiota of young children in Sweden was found to contain a relatively large number of *Clostridium* spp., whereas high levels of *Lactobacillus* spp. and *Eubacterium* spp. were detected in an age-matched population from Estonia;²⁵⁵ this difference might contribute to the lower incidence of allergy in the Baltic countries compared with Scandinavia.²⁵⁶ A Finnish study likewise reported that allergic infants had more Clostridia and tended to have fewer bifidobacteria in their stools than non-allergic controls.²⁵⁷ The impact of differences in diet on this commensal variability remains undefined.¹³³

Absence of early postnatal gut colonization with a normal commensal microbiota dominated by lactic acid-producing bacteria probably contributes to the increased risk for food allergy generally noted in children delivered by cesarian section, particularly when genetically predisposed.^{124,125} Nevertheless, in clinical studies it has been difficult to reveal a convincing effect on oral tolerance by probiotic perinatal intervention, even in children at high risk for food allergy.²³⁰ The same conclusion was reached with regard to IgE-mediated allergy at the age of five years (including food allergy) after extending the postnatal intervention (four probiotic strains combined with prebiotics) until the age of six months, as observed in an extensive Finnish study.²⁵⁸ However, for children delivered by cesarian section, a modest but significant allergy reduction was noted when they were randomized to the same regime.²⁵⁸ Also, intestinal colonization of lactobacilli and bifidobacteria is promoted by breastmilk because it acts as prebiotics through its large amounts of oligosaccharides,^{40,158} and it may also contain probiotic bacteria.^{165,259}

Thus, there is hope for clinical benefits by balancing the colonization of the gut microbiota and inducing homeostatic immune regulation, but a molecular approach by identifying and applying the active MAMPs would be desirable. An alternative promising approach is fecal transplantation (transfer of feces from a healthy person) to treat microbial imbalance, or dysbiosis.^{133,260} However, live bacteria can show phase-variable expression of carbohydrate determinants on LPS which interacts with C-type lectin receptors such as DC-SIGN; integration of these receptors with other PRRs may therefore modulate the level of NFκB activation *versus* an anti-inflammatory response.²⁶¹

The feeding and treatment conditions (e.g., antibiotics) to which the newborn is subjected, and also the general nutritional state, may have an impact on the composition of the indigenous microbiota as well as on the epithelial integrity;¹³³ such variables may hence contribute to the programming of the mucosal immune system.^{262,263} Lipid intake, such as fish oil enriched with polyunsaturated omega-3 fatty acids, may protect against food allergy, but apparently not against established allergic disease.²⁶⁴ Fecal levels of SCFAs have been shown to be relatively low in children with food allergy; this apparently reflects a slow maturation of the gut microbiota.²⁶⁵

Lipid-based allergy prevention strategies should, therefore, be performed early, perhaps even *in utero*.²⁶³ Animal experiments have suggested that the ratio of omega-6:omega-3 fatty acids is of particular importance for neonatal oral tolerance induction.²⁶⁶ This ratio varies in breastmilk from women in different parts of the world, a finding that may explain the reported variable effects of breastfeeding on allergy prevention. A bioactive derivative of omega-3 fatty acids, termed resolvin E1, binds with high affinity to a G protein-coupled receptor named ChemR23 which is expressed on APCs; such signaling attenuates NFκB activation.²⁶⁷ Another G protein-coupled receptor named GPR120 is a direct sensor of omega-3 fatty acids and induces broad and robust anti-inflammatory effects in experimental animals.^{133,268} These pathways together may explain, at least in part, the anti-inflammatory effects of omega-3 fatty acids. Finally, cell culture studies have suggested that probiotics could be directly immunomodulatory by enhancing the Th1 profile via induction of IL-12, IL-18 and IFN-γ secretion.^{269,270} Also notably, *E. coli* is a strong inducer of IL-10 secretion, apparently derived both from APCs and Treg cells.^{271,272} Importantly, Treg cells bear PRRs for several MAMPs,²⁷³ and IL-10 is crucial for maintained expression of the Foxp3 transcription factor,²⁷⁴ which contributes significantly to the suppressive function of these cells.²⁰⁸ Interestingly, IL-10 has been directly shown to be an important suppressive cytokine in the murine gut.²⁷⁵

The above information collectively implies that the gut microbiota may tailor mucosal immunity beyond that of enhancing the SIgA system, namely by promoting a balanced development of Th1, Th17, Th2 and Treg cells. As mentioned previously, however, the selection of safe and effective probiotic strains remains difficult,^{230,232} and the impact of the diet on gut microbiota and homeostasis must always be considered.¹³³

2.7 Conclusion

A number of biological variables influence induction of oral tolerance and SIgA-dependent intestinal immunity (Fig. 2.14). Increased epithelial permeability for exogenous antigens is clearly an important primary or secondary event in the pathogenesis of several disorders affecting body surfaces. The gut barrier function is determined by the individual's age (e.g., preterm *versus* term infant), diet, genetics, mucus composition, interactions between mast cells, nerves and neuro-

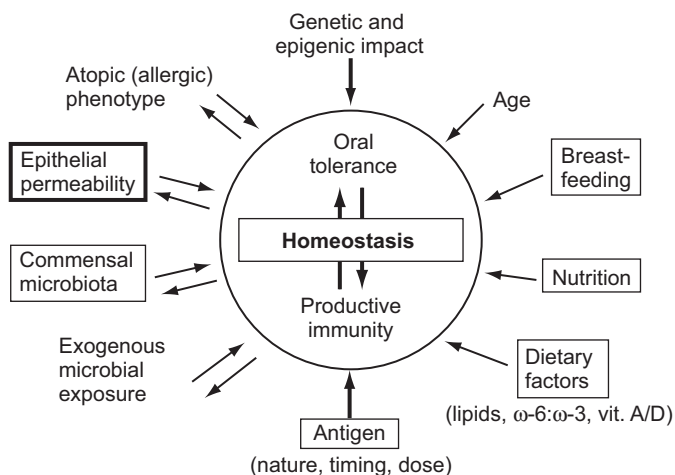


Fig. 2.14 Biological variables with an impact on the developing immunophenotype of the infant. Immunological homeostasis depends on the balance between mucosally induced oral tolerance and productive immunity including secretory IgA antibodies and systemic type of pro-inflammatory immune mechanisms. Several of the components acting on this balance are reciprocally modulated as indicated by bidirectional arrows. Framed variables represent biological impacts that may be subjected to various intervention modalities as discussed in the text. The importance of the epithelial barrier function is highlighted by a bold frame (see Figs 2.8 and 2.10).

peptides, concurrent infection, the commensal microbiota and the mucosa-shielding effect of SIgA provided by breastmilk or produced in the infant's gut. The integrity of the epithelial barrier furthermore depends on homeostatic regulatory mechanisms, including mucosal induction of Treg cells, where commensal microbial–host interactions apparently play decisive roles. There is currently considerable interest in how extrinsic and intrinsic variables impact on the intercellular tight junctions and thereby on the epithelial barrier function in the gut.²⁷⁶

The secretory immune system is critical for the mucosal barrier because SIgA not only forms the first line of adaptive immune defense but also maintains mutualism with the indigenous microbiota. In mouse experiments it has indeed been shown that a single immunomodulatory molecule from a commensal gut bacterium can induce crucial modulation and homeostasis of the host's immune system.^{152,153} Also notably, the epithelial barrier depends on exposure to microbial components (MAMPs) from the commensal microbiota and the environment, both by direct interaction with PRRs on the intestinal epithelium and by induction of oral tolerance via mechanisms such as tolerogenic APCs and Treg cells.

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80 Diet, immunity and inflammation

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3

Chronic inflammatory diseases

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Abstract: For several decades the incidence and prevalence of chronic inflammatory diseases have been increasing, particularly in westernized countries. These diseases include allergic conditions (asthma, eczema) as well as autoimmune diseases. It is now well established that the development of clinical phenotypes is the result of an intimate interaction between genetic predisposition and environmental exposures. The adaptive immune system plays an important role in orchestrating the inflammatory response. In this regard, T-helper cell differentiation into Th1, Th2, Th17, Treg and other T-cell subsets plays an important role. Recent data further indicate that there is a high degree of flexibility and plasticity among these effector cells. T-helper cell differentiation is tightly controlled by epigenetic mechanisms. These include DNA methylation, histone acetylation and the role of microRNA. Epigenetic regulation represents an important mode of action for environmental factors – including nutritional agents, stress and microbial compounds – to regulate gene expression resulting in disease development.

Key words: chronic inflammation, T-helper differentiation and plasticity, epigenetic chromatin modifications, microRNA.

3.1 Introduction

Medical conditions described as chronic inflammatory diseases represent a Janus-faced complex of pathogenetic trails. On the one hand, these conditions are based on a common thread characterized by a persistent inflammatory response that can wax and wane, and an inappropriate or deviated response of the immune system that is already determined in early life and often resists treatment. On the other hand, the diseases gathered under this umbrella differ in many features (Dowrick *et al.*, 2005; Smith, 2011). Chronic inflammation can affect nearly all organs and

tissues and thereby lead to a variety of symptoms and natural disease courses. Although all chronic inflammatory conditions are associated with considerable damage to host tissues, the pathological processes and the symptomatic manifestations appear highly divergent, depending on the organ that is targeted by the process and the type of inflammation (granulomatous, fibrinous, serous, ulcerative, etc.) that damages the organ. Interaction with, and effects on, other organ systems may complicate and diversify the individual outcome of a patient and, in the end, may lead to a spread of inflammation within the body. Basically, inflammation is a protective response of the body against damaging insults, infectious agents and injuries that initiates healing, but its chronification compromises even these healing processes (Nathan, 2002).

Chronification of the inflammation and persistence and recurrence of symptomatic manifestations are associated with a high burden of disease. Life-long medical histories imply a great deal of suffering and a detriment of life quality for the individual patient and result in a loss of social and economical efficiency with consequences on the societal level. The lack of proper effective medications for many of these disorders may contribute to the high individual and socio-economic burden that occurs (Yach *et al.*, 2004). This chapter will give an overview on the epidemiological trends that have been observed in recent decades and on the basic mechanisms of chronic inflammatory diseases.

One common thread that connects many chronic inflammatory diseases is an inappropriate immune response. The immune system is the major construct by which organisms act in the defence of hazardous and infectious agents. Furthermore, tolerance mechanisms established early in life regulate the recognition of allo- or xenogenic and autologous cellular compartments. Disruptors in the natural development of tolerance seem to mark the onset of chronic inflammatory processes (von Hertzen *et al.*, 2011). Deviation in the programming of immune cells may lead to allergic reactions against harmless environmental factors or may result in an autoimmune reaction that is characterized by an immunological attack directed at the host's own cells and tissues. These overwhelming reactions are followed by inflammatory processes in the affected organ. The various sections in this chapter aim to provide an overview on current status of the immunological mechanisms that contribute to chronic inflammation and thereby to chronic inflammatory diseases.

The origins of chronic inflammatory diseases are not fully understood. Some of these, such as cystic fibrosis, are Mendelian disorders determined by a clear genetic defect transmitted congenitally, but the majority of chronic inflammatory conditions are based on the involvement of certain multiple genes that contribute to the risk of developing such disorders (Kere, 2010; Szic *et al.*, 2010). Nevertheless, in most cases the onset seems to be unpredictable and random. Therefore, prediction of further exacerbations and severity is not yet possible. In many cases, the lack of reliable and disease-specific biomarkers that allow for an early identification of individuals at increased risk may contribute to this situation. Additionally, the fact that most of these disorders are influenced or triggered by environmental factors complicates the understanding of the underlying causative

mechanisms. Undoubtedly, genetic background contributes to these conditions but, in addition, lifestyle factors, diet, environmental exposures and socio-economic factors may interfere with genetic signatures and shape the individual onset and course of the disease, probably via epigenetic modifications (Wilson, 2008). Interactions between the environment and the genetic background gain more and more attention in the basic field of research as well as in epidemiological studies. This chapter aims to present this new and exciting field and provides an outlook on future implications.

3.2 Epidemiological trends of chronic inflammatory diseases

The spectrum of chronic inflammatory diseases is broad and ranges from highly prevalent disorders to orphan diseases. The most common disorders such as rheumatoid arthritis (RA), allergies and asthma, and chronic obstructive pulmonary disease (COPD), associated with markers of ongoing inflammation are widespread in the affluent nations. Since the 1950s, a significant increase of chronic somatic disorders has been observed while incidences of common infectious diseases decreased in the same parts of the world (Bach, 2002). These inverse trends observed for communicable and non-communicable diseases in the industrial countries are associated with changing individual and social living conditions and environments. Westernized societies are characterized by a diminished exposure to pathogenic and non-pathogenic microbial stimuli. These stimuli seem to play a crucial role in the development of tolerance mechanisms that enable the immune system to distinguish between pathogenic, commensal and benefiting microbes. The absence of these stimuli may result in pathogenic conditions that favour initiation and chronification of inflammatory processes. The deviation of the immune system that contributes to a loss of tolerance and the development of allergies and autoimmune diseases and the lack or dysfunction of regulatory pathways that terminate inflammation act to promote the further development of chronification (von Mutius, 2010).

Numerous epidemiological studies, specifically those conducted in a traditional farm environment ‘model situation’, indicate that the underlying mechanisms of tolerance are established in very early life. Priming of immunoregulation seems to be restricted to a close ‘window of opportunity’ from the late prenatal phase of development to the second year of life (von Mutius and Radon, 2008). Translation of these ideas into an experimental murine model of prenatal immune modulation strongly supported the epidemiological hypothesis of early immune programming (Conrad *et al.*, 2009).

3.3 T-helper cells: differentiation and effector cell plasticity

Chronic inflammatory diseases, evoked by a variety of different pathogens and agents, are orchestrated by specialized effector T-cells. Under the control of

genetic programs, antigen-inexperienced, naïve CD4⁺ T-cells (Th0) differentiate to various T-helper subsets upon encountering antigens, depending on signals that innate immune cells provide in a cognate and non-cognate fashion. These programs coordinate the specialization process by inducing different transcription factors for the expression of distinct molecules to support the interaction with other immune cells. With the secretion of soluble mediators and expression of surface molecules, T-helper (Th) cells provide help for B lymphocytes and CD8⁺ cytotoxic T-cells and activate innate immune effector cells, including macrophages, neutrophils, eosinophils and mast cells, all targeting distinct pathogens, and assuring successful immune defence responses. Th1, Th2, Th17, regulatory T-cells, Tfh, Th22 and Th9 are defined based on the expression of distinct transcription factors and the production of a panel of cytokines.

3.3.1 T-helper cell differentiation

Th1 differentiation is induced by interferon- γ (IFN- γ) and interleukin (IL)-12 and downstream activation of signal transducer and activator of transcription 1 (STAT1) and STAT4 and induction of the Th1-specific transcription factor T-bet, also known as Tbx21. Th1 cells produce IFN- γ and pro-inflammatory cytokines, including tumour necrosis factor α (TNF- α) and TNF- β . Their prime function is the activation of macrophages and protection from obligate intracellular pathogens by stimulating cell-mediated immunity defined by cellular cytolytic activity. Exuberant activity of Th1 cells is associated with tissue damage, and triggers self-reactivity and undesired inflammatory diseases as observed in inflammatory bowel disease, autoimmune and RA.

Th2 cells differentiate upon IL-4-induced activation of STAT6 and subsequent induction of the master transcription factor GATA-binding protein 3 (GATA-3). The expression of GATA-3 is sufficient and required for Th2 differentiation. Th2 cells produce IL-4, IL-5, IL-10 and IL-13 as marker cytokines and are critical for immunoglobulin (Ig) E production, eosinophil recruitment, clearance of extracellular pathogens and mucosal immunity. Aberrant activation of Th2 cells can cause allergic reactions, including bronchial asthma.

Th17 cells differentiate from naïve T-helper cells by the combined action of transforming growth factor β (TGF- β) and IL-6 and activation of STAT3. They express the retinoic acid-related orphan receptors (ROR) - α and - γ t as key transcription factors. As marker cytokines, Th17 cells produce IL-17A and F, IL-21 and IL-22 and play a critical role in the induction and propagation of autoimmune diseases, including experimental autoimmune encephalitis (EAE), RA, psoriasis, inflammatory bowel disease and allergic responses. They are also important in immune responses against extracellular bacteria and fungi.

Regulatory T (Treg)-cells can either develop in the thymus (naturally occurring Treg; nTreg) or differentiate from naïve T-cells in the presence of TGF- β and downstream activation of STAT5 following T-cell receptor stimulation (induced Treg; iTreg). nTreg are characterized by the expression of forkhead-box-protein 3 (Foxp3) as a master transcription factor, which controls the expression of a wide

variety of genes including cytokines and surface molecules. In contrast to other T-helper subsets, which promote immune responses, Treg cells are important for dampening immune responses and are therefore anti-inflammatory. Their main function is to maintain self-tolerance and preserve immune homeostasis. A disruption of Treg cell function causes a plethora of autoimmune and inflammatory pathologies.

Follicular helper T (T_{fh})-cells are found in B-cell follicles, where they provide help for the development of germinal centres and promote immunoglobulin class switch recombination and affinity maturation in response to T-cell-dependent antigens (King *et al.*, 2008; Zhou *et al.*, 2009). As their master transcription factor T_{fh} express B-cell lymphoma 6 (Bcl-6) which, unlike T-bet and GATA-3, is a transcriptional repressor (Johnston *et al.*, 2009; Nurieva *et al.*, 2009). They produce large amounts of IL-21 which acts, in a Bcl-6 dependent process, in an autocrine manner with IL-6 on their differentiation and expansion (Vogelzang *et al.*, 2008; Nurieva *et al.*, 2009; Yu *et al.*, 2009). T_{fh} can be distinguished from Th1 and Th2 cells by several criteria. They continuously express the chemokine receptor CXCR5, which makes them, unlike other migratory T-helper cells, reside in B-cell follicles and support antibody production more efficiently. Although T_{fh} cells have distinct properties and phenotypes, it is unclear whether they represent a distinct subset, since activated CD4⁺ T-cells transiently express CXCR5 and individual T_{fh} cells may produce Th1 or Th2 cytokines, depending on the condition of their initial priming (King *et al.*, 2008; Zhou *et al.*, 2009).

Th22 cells were recently identified in humans, but not mice, and represent an inflammatory T-helper subset, like Th17 cells, which expresses IL-22 but not IL-17 or RoR γ t. Th22 differentiation is induced by aryl hydrocarbon receptor (AhR) ligands. These cells home to the skin and are responsible for the inflammation observed in psoriasis, for example. Th22 cells preferentially develop when cultured in the presence of plasmacytoid dendritic cells, which infiltrate psoriatic skin.

Complexity of T-cell subsets further increased with the identification of a distinct population of CD4⁺ T-cells, which depends on IL-4 and TGF- β for their development *in vitro*. These cells activate STAT6, express the transcription factor PU.1 and secrete IL-9, a cytokine once considered as Th2-specific. It is now evident that IL-9 is co-expressed neither with IL-4, IL-5 or IL-13 nor by other T-cell subsets like nTreg or iTreg. Yet, as this population has only been studied *in vitro*, it is unclear whether IL-9-producing T-cells constitute a distinct helper cell population, or whether expression of this cytokine reflects adaptation of Th2 cells to a changed micro-environment in the progression of a disease triggered by pathogens or allergens (Fig. 3.1).

3.3.2 Flexibility and plasticity of effector cell subsets

Classically, Th cells were believed to represent terminally differentiated cell lineages, which were committed to a certain path based on the genetic program activated. This hypothesis was strengthened by the finding that activated genetic programs provide self-reinforcing feedback circuits, as shown for T-bet and

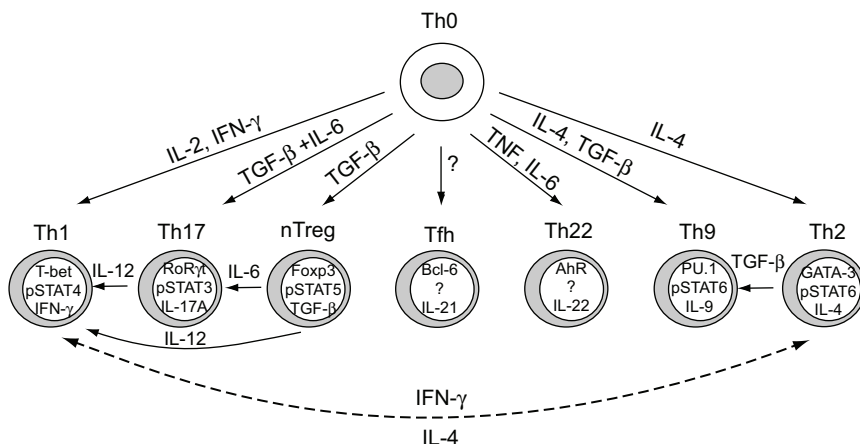


Fig. 3.1 T-cell differentiation and plasticity.

GATA-3, and the observed transcriptional interference between transcription factors, where one factor neutralizes the transcriptional activities of the other. However, accumulating *in vitro* and *in vivo* evidence supports the concept of functional flexibility and plasticity in Th cell subsets. This might be attributable to the heterogeneity of each expanding Th cell population, comprising cells at diverse differentiation and commitment stages, the duration of antigen stimulation, a changed micro-environment and the interaction with innate immune cells affecting the genetic program.

Flexibility in the genetic program can be observed in all Th cell subsets. In addition to the expression of different transcription factors, a mixed and overlapping cytokine profile expression can be observed when cells encounter a changed micro-environment. For Th1 cells, the expression of IL-4, like the expression of IFN- γ in Th2 cells, could be induced by a modified short-term culture condition, and even IFN- γ ⁺IL-4⁺ double positive cells could be detected. IL-17-producing cells can easily acquire the capacity to produce IFN- γ in a STAT1- and T-bet-independent fashion. Furthermore, the existence of IFN- γ ⁺IL-17⁺ cells was documented *in vivo* (Lee *et al.*, 2009). Treg cells as well, although documented as anti-inflammatory, can interchange into other inflammatory subsets. When Treg are exposed to Th1 conditions *in vitro* or *in vivo*, they upregulate the expression of T-bet and are able to produce IFN- γ . When Treg cells are exposed to IL-6, which is critical for Th17 differentiation, ROR γ t expression and IL-17 production are induced. Likewise, the existence of IL-17-producing Foxp3⁺ cells is documented in mice and humans. While for all subsets reprogramming after short-term culture is documented, fully differentiated Th1 and Th2 cells seem to obtain a stable state. Yet, it seems that the longer the cells are exposed to a certain micro-environment and the further the cells differentiate, the harder it is to alter their genetic program. Furthermore, distinct subsets, like Foxp3⁺ Treg and Th17 cells seem to be unstable or plastic throughout their differentiation process, unlike Th1 and Th2 cells.

The ability of T-helper cells to change their phenotype from one effector-type to another upon encountering an altered micro-environment implies a certain degree of flexibility in their genetic program. This flexibility enables T-helper cells to modify the expression pattern of cytokines adapted to the ongoing immune reaction. In chronic inflammatory diseases, an augmented expression of a variety of effector molecules by T-helper cells is observed. In this context, several cellular mechanisms have been identified regulating gene expression. These mechanisms can either interfere at the transcriptional level or regulate gene expression post-transcriptionally. Examples for such regulatory mechanisms are epigenetic modifications and microRNA.

3.4 Chronic inflammation and epigenetics

In recent years it has become more and more obvious that the genetic and the epigenetic information provided by the cells of an organism have a strong influence on the onset and course of chronic inflammation. An inherited susceptibility for several diseases associated with chronic inflammation like Crohn's disease, multiple sclerosis (MS) and allergic asthma has been described in a wide variety of studies (Hugot *et al.*, 2001; Oksenberg and Baranzini, 2010; Sleiman and Hakonarson, 2010). In addition to the information yielded by the DNA sequence itself, epigenetic modifications of the chromatin affect the transcriptional regulation of a certain gene and thus strongly influence the phenotype. Epigenetic modifications are chromatin modifications which do not change the DNA sequence, but modify the chromatin structure and alter the accessibility of the DNA for transcription factors and co-factors. These modifications can be mitotically inherited from one cell to another, and some of them might also be stably inherited into the next generation (transgenerational inheritance of epigenetic marks through meiosis) (Chong and Whitelaw, 2004). Nevertheless, even if epigenetic marks can be inherited, they can also be changed in response to stimuli coming from the environment. This mechanism provides a certain flexibility to the genetic read-out of cells and organisms.

That environmental inputs can strongly influence the susceptibility for several diseases already in early phases of life has been described by the developmental origins of health and disease (DOHaD) hypothesis. This hypothesis proposes that during critical periods of pre- and postnatal development, environmental stimuli like nutrition can induce changes in metabolism and in chronic disease susceptibility. It emerged when it became evident that, for example, low birth weight is associated with an increased risk for cardiovascular disease, adiposity and type 2 diabetes (metabolic syndrome) (Barker *et al.*, 1989, 1993). Low birth weight can be caused by genetic factors, but can also occur due to environmental factors, including under-nutrition and smoking by the mother, causing an impaired intra-uterine milieu. It is suggested that impairment of the intrauterine milieu leads to a metabolic reprogramming that might be beneficial in the short term for survival, but might be harmful for the organism in the long run (thrifty phenotype hypothesis

or Barker hypothesis). In the case of maternal under-nutrition the developing foetus may develop an insulin resistance in order to conserve energy during the time of under-nutrition. This *in utero* adaption promotes survival in times of nutritional deprivation, but can lead to development of the metabolic syndrome if the postnatal environment conditions do not match the prenatal conditions (match–mismatch principle) (Gluckman *et al.*, 2008). Besides nutrition, environmental stimuli such as the exposure to microbes and parasites during pre- and postnatal development can influence the susceptibility to chronic disease like allergies and asthma (hygiene hypothesis) (Garn and Renz, 2007).

Reprogramming of the fetal metabolism and/or immune system in response to environmental stimuli during certain ‘windows of opportunity’ requires a developmental and transcriptional plasticity which can be provided not by the genome but by the epigenome. Unlike the DNA sequence, epigenetic modifications of chromatin by DNA methylation and histone modifications can be changed in response to environmental stimulation to alter the gene expression pattern and to optimize the response of an organism to its environment. Interestingly, mammals are particularly susceptible to changes in the epigenetic code during the pre- and perinatal period (Jirtle and Skinner, 2007).

Nevertheless, even if the pre- and perinatal period seem to be critical for setting up the epigenome, epigenetic changes can also occur in later stages of life. The process of ageing itself also modifies the epigenome of cells, thus leading to changes in gene expression. This seems to be especially important for the development of certain cancers as epigenetic changes might lead to a repression of tumour suppressor genes and to an enhanced expression of oncogenes.

3.4.1 Epigenetic mechanisms

There exist distinct mechanisms to change the accessibility of chromatin for the transcriptional apparatus – the modification of the DNA itself and the modification of histone proteins which set up the chromatin together with the DNA. The most common epigenetic DNA modification is the methylation of cytosines in the context of CpG-dinucleotides. Through the recruitment of methyl DNA-binding proteins, transcription factors and chromatin remodelling complexes, the methylation status of the DNA can influence the transcription of a gene. In general, methylation leads to gene silencing whilst hypomethylation goes along with transcriptional activation. Promoter regions of several mammalian genes are especially enriched with CpG dinucleotides building up so-called CpG islands which seem to provide essential information for the expression of a gene by their methylation status.

While the DNA sequence is the same in every cell of an organism, the methylation pattern can vary between distinct cell types and eventually lead to distinct gene expression patterns. To set up and to maintain the methylation patterns, mammalian cells express distinct DNA methyl transferases (DNMTs). DNMT1, the so-called maintenance methyl transferase, is needed to methylate the newly synthesized DNA strand in the same pattern as the template strand after

DNA replication. In addition, DNMT3a and DNMT3b are *de novo* methyl transferases and are needed to methylate previously unmethylated DNA to create new methylation patterns.

DNA demethylation can very likely be a passive as well as an active process. Passive DNA methylation occurs during DNA replication in the absence of DNMT1. The mechanisms of active DNA demethylation are less well understood, yet the contribution of several mechanisms, e.g. enzymatic removal of the methyl group, base excision repair and oxidative demethylation, to active demethylation is proposed (Wu and Zhang, 2010).

These mechanisms are especially important during embryogenesis when, shortly after fertilization, the genome of the early embryo undergoes a massive demethylation correlating with the pluripotency of the embryo. At the time point of implantation, *de novo* DNA methylation starts to re-establish lineage-specific DNA methylation. These periods of loss and re-establishment of DNA methylation are believed to be especially prone to environmental stimuli that can affect epigenetic regulation (Waterland and Jirtle, 2004).

A wide variety of histone modifications can influence the chromatin structure by leading to an ‘open’ or a more ‘closed’ chromatin structure, thereby allowing (permissive chromatin structure) or preventing (repressive chromatin structure) gene transcription. The best characterized histone modifications are acetylation and methylation of histone proteins. Histone acetylation normally leads to a permissive chromatin structure, while histone methylation can be permissive as well as repressive, depending on the nature of the modification itself (Sadri-Vakili and Cha, 2006). Beside these modifications, histones can be phosphorylated, ubiquitinated and sumoylated (Table 3.1). The different combinations of histone modification set up a ‘histone code’ which strongly influences gene expression of distinct cell types. The histone code is established and modified by several histone-modifying enzymes like histone acetylases (HATs), histone deacetylases

Table 3.1 Transcriptional repressive and permissive chromatin modifications in mammals

Modification			Permissive	Repressive
DNA methylation (cytosine)				+
<i>Histone modification</i>		<i>Amino acid</i>		
Acetylation	H2A	K5	+	
	H2B	K5, K12, K15, K20	+	
	H3	K4, K9, K14, K18, K23, K27	+	
	H4	K8, K16	+	
Methylation	H3	K4, K79, R17	+	
		K9, K27		+
	H4	R3	+	
		K20		+
Ubiquitination	H2A	K119		+
	H2B	K120	+	

(HDACs) and histone methyl transferases (HMTs). DNA methylation and histone modifications are very closely linked to each other since it has been shown that methyl DNA-binding proteins can recruit histone-modifying enzymes to the DNA while vice versa certain histone modifications can influence the methylation status of the DNA (Cheng and Blumenthal, 2010).

3.4.2 Epigenetic regulation of T-helper cell differentiation

Chronic inflammation develops when an acute inflammatory stimulus persists over a longer period of time than it should. This occurs when the immune system is not able to remove the stimulus, e.g. when the stimulus is an environmental antigen (allergy) or in the case of autoimmunity. Immune reactions during distinct types of acute and chronic inflammation are driven by distinct T-helper cell subsets (Jager and Kuchroo, 2010). Differentiation of these T-helper cells and thus the immune response itself are important targets of epigenetic regulation (Janson *et al.*, 2011b) and so one can easily imagine that the epigenetic programming of the immune system can strongly influence the course of chronic inflammatory disease.

The contribution of epigenetic changes going along with the differentiation of the distinct T-cell subsets has been in the focus of the investigation in recent years. The previously mentioned Th1, Th2, Th17 and Treg cell subsets are generally known to be involved in several chronic inflammatory diseases. As described before, these T-cell subsets are characterized by the expression of transcription factors T-bet (Th1), GATA-3 (Th2), Foxp3 (Treg) and RoR γ t (Th17) as well as by the expression of signature cytokines like IFN- γ (Th1), IL-4 (Th2) and IL-17 (Th17). The expression of these genes during T-cell differentiation and inflammation is regulated by DNA methylation and several histone modifications.

Th1 cells are very important during acute immune responses since the resolution of bacterial and viral infections strictly depends on this cell type. In contrast to these beneficial functions of Th1 cells, they are also believed to drive several types of disease characterized by chronic inflammation such as RA and MS. The main cytokine secreted by Th1 cells and one of the most important mediators of chronic inflammation is the pro-inflammatory cytokine IFN- γ .

To establish and maintain IFN- γ expression during Th1 cell differentiation and proliferation, the gene locus undergoes a plethora of epigenetic modifications, which are partially regulated by T-bet. During Th2 cell differentiation, the methylation status of the IFN- γ promoter changes significantly. In naïve murine T-cells the IFN- γ promoter is hypomethylated, and this remains unchanged under Th1-polarizing conditions. In contrast, under Th2-polarizing conditions, the IFN- γ promoter is a target of *de novo* methylation at several positions, which seem to be at least partially conserved during evolution. The methylation of the locus has a significant impact on the IFN- γ transcription level; very likely by interfering with binding of transcription factors like cAMP response element binding protein (CREB) and activating transcription factor (ATF2)/c-Jun to their target sites (Jones and Chen, 2006). Additionally, Th1 and Th2 cells exhibit distinct patterns

of histone modification of the IFN- γ locus, which can mediate transcriptional activation or silencing of the IFN- γ gene. Acetylation of histones H3 and H4 is clearly linked to a transcriptional permissive (open) chromatin structure, and it has been shown that during T-cell polarization the IFN- γ promoter becomes hyperacetylated in Th1 cells but not in Th2 cells (Fields *et al.*, 2002).

Dimethylation of lysine 4 of histone 3 (H3K4me2) is a classical permissive histone mark, while trimethylation of lysine 27 of histone 3 (H3K27me3) is associated with transcriptional silencing. Interestingly, the Th1 transcription factor T-bet can recruit H3K4me2-methyltransferase activity to target promoters as well as being able to mediate the removal of repressive H3K27me3 modifications. This might provide an explanation for the increased rate of H3K4me2 methylation and the decreased rate of H3K27me3 methylation observed in Th1 cells *versus* Th2 cells and naïve T-cells (Schoenborn and Wilson, 2007), ultimately making the IFN- γ locus accessible for the transcription machinery more or less exclusively in Th1 cells.

Th2 cells are crucial for the defence of the body against extracellular bacteria and parasites. Nevertheless, it has been clearly reported that Th2 cells are also the mediators of allergic asthma, a chronic inflammatory disease of the respiratory tract, and of other atopic diseases. Crucial for the function of Th2 cells is the expression of the Th2 cytokines IL-4, IL-5 and IL-13 from the Th2 cytokine locus containing the coding and regulatory regions for all three cytokines and for *Rad50*. The DNA at this locus is hypermethylated in naïve T-cells and becomes progressively demethylated during Th2 cell differentiation due to decreased maintenance methylation during cell division. The histone modification pattern of the Th2 locus also changes significantly upon T-cell activation and polarization. In naïve T-cells the locus is in a silent but poised state for the transcription of IL-4 and IL-13 with both permissive and silencing histone modification at the locus. Upon activation of naïve T-cells, early IL-4 transcription occurs accompanied by permissive chromatin modifications like histone H3 acetylation in Th1 cells as well as in Th2 cells (Fields *et al.*, 2002; Baguet and Bix, 2004). In Th2 cells the permissive state characterized by H3 and H4 acetylation and H3K4me2 becomes stabilized while in Th1 cells the permissive modifications are erased and replaced by repressive modifications like H3K27me3 (Koyanagi *et al.*, 2005). Eventually, the combination of differential DNA methylation and distinct histone modifications at the Th2 cytokine locus leads to stable expression of Th2 cytokines by T-cells under Th2-polarizing conditions, while in Th1 cells these genes are transcriptionally silenced (Ansel *et al.*, 2006).

Treg counter-regulate (suppress) effector T-cells and are, for example, crucial for establishment of self-tolerance, the suppression of allergy and asthma and suppression of T-cell activation by weak stimuli (Corthay, 2009). Treg cells usually express the transcription factor Foxp3. Defects in the Foxp3 gene can lead to a severe dysfunction of the immune system in mice and humans due to the lack of Treg cells (Torgerson and Ochs, 2007). DNA methylation seems to play an important role for the establishment and stabilization of the Treg lineage. The *foxp3* promoter contains a region called Treg-specific demethylated region (TSDR),

which is highly conserved during evolution and strikingly 100% methylated in CD4⁺CD25⁻ T-cells, whilst completely demethylated in naturally occurring CD4⁺CD25⁺ Treg cells. It has been demonstrated that demethylation of the TDRS regulates Foxp3 transcription and is also critically for maintaining stable Foxp3 expression and thus promotes stable lineage commitment of Tregs. Additionally, in CD4⁺CD25⁺ cells the *foxp3* locus is marked by an increase in histone modifications, which facilitate an open and transcriptional permissive chromatin structure like H3 acetylation and H3K4 trimethylation (Floess *et al.*, 2007; Polansky *et al.*, 2008).

Currently, there is only limited knowledge on the contribution of epigenetic regulation to differentiation and function of other T-cell types like Th17 cells, which are also involved in the development of autoimmune disease accompanied by chronic inflammation like RA (Peck and Mellins, 2009). Demethylation of the promoter of the *IL17A* gene seems to promote the expression of IL-17A by Th17 cells and it has been shown that IL-17 transcription during Th17 differentiation is also regulated by the acetylation of histone H3 and by trimethylation of H3K4 (Akimzhanov *et al.*, 2007; Janson *et al.*, 2011a).

3.4.3 Chronic inflammatory diseases and epigenetic changes

One prime example for a chronic inflammatory disease that seems to depend on the ratio of Th1 to Th2 cells is allergic asthma. A strong bias towards the Th2 cell type after activation of T-cells following allergen inhalation drives immediate type I hypersensitivity and can eventually lead to chronic inflammation of the lung. The development of this disease is strongly dependent on exposure to several environmental factors, especially during childhood and, intriguingly, also during the prenatal period. An exposure to airborne pollutants like environmental tobacco smoke, diesel exhaust particles or particulate matter in the pre- and perinatal period increases the risk of developing allergic asthma, while the confrontation of the immune system with certain microbes (e.g. in a traditional farming environment) protects from asthma development. As environmental inputs are able to reprogram the epigenome, it is very likely that the susceptibility to develop allergic asthma as a result of environmental exposures is dependent on epigenetic modifications of T-helper cells. That reprogramming of the epigenome of Th cells does indeed occur in response to environmental factors has been demonstrated in experimental mouse models of allergic asthma. The exposure of mice sensitized with *Aspergillus fumigatus* to diesel exhaust particles leads to a hypermethylation of the IFN- γ promoter, while the IL-4 promoter becomes hypomethylated at one specific site. These changes in DNA methylation go along with an altered IgE production (Liu *et al.*, 2008).

During the prenatal period, maternal diet can directly modify CpG methylation and the asthmatic phenotype. This has been shown by feeding pregnant mice with a methyl donor-rich diet. The offspring of these mice exhibited an enhanced asthmatic reaction after OVA challenge even in the F2 generation and increased site-specific DNA methylation. In this case, changes in DNA methylation did not

occur at the promoters of the Th1 or Th2 cytokines directly, but affected for example the methylation of Runx3. Interestingly, Runx3-deficient mice spontaneously develop an asthma-like phenotype which might be due to the cooperation of Runx3 with T-bet to silence IL-4 expression. The change in DNA methylation leads to a reduced Runx3 expression so that the observed skewing towards a Th2 phenotype might be due to increased IL-4 expression (Hollingsworth *et al.*, 2008).

In humans, prenatal exposure to a farming environment can significantly decrease the risk of allergic diseases in the offspring. Interestingly, the analysis of Treg cells taken from cord blood revealed an increase in Treg numbers and the suppressive capacity of the Tregs from farming mothers. Additionally, a significant decrease of *foxp3* methylation after exposure to farm milk was detected, suggesting that prenatal epigenetic modifications can influence the allergic phenotype in humans also (Schaub *et al.*, 2009).

In addition to alteration of the DNA methylation, changes of the histone code might also influence the asthmatic phenotype. For example, the treatment of OVA sensitized and challenged mice with the histone deacetylase inhibitor trichostatin A (TSA) led to decreased expression of Th2 cytokines and an attenuated airway inflammation (Choi *et al.*, 2005). Nevertheless, even if this observation suggests that changes in histone acetylation are involved in the transcriptional regulation of Th2 cytokines, this could be also a secondary effect. TSA is known to interfere with the phosphorylation of the nuclear factor kappa B (NFκB) subunit p65, thus inhibiting inflammatory reactions mediated by NFκB (Niu *et al.*, 2009), a factor which critically regulates GATA-3 expression and Th2 differentiation (Das *et al.*, 2001).

Apart from epigenetic regulation of the expression of certain cytokines and transcription factors involved in differentiation and function of different T-helper cell subsets, several special patterns of epigenetic marks can be associated with chronic inflammatory disease. Patients with systemic lupus erythematosus (SLE) exhibit a global hypomethylation of their T-cell DNA. The exposure of T-cells to demethylating drugs leads to the induction of lupus-like disease in mice, showing that global DNA demethylation in T-cells can indeed induce SLE. The mechanism for this induction of SLE due to DNA hypomethylation is not completely understood, but the DNA demethylation leads to the over-expression of several genes like CD11a, CD70 and perforin involved in the pathogenesis of SLE. The reason for the DNA hypomethylation in this disease might be a downregulation of DNMT1 caused by impaired extracellular signal-regulated kinase (ERK) signalling of lupus T-cells. Interestingly, in humans the drugs hydralazine – an inhibitor of ERK signalling – and procainamide – a DNMT1 inhibitor – can be a cause of drug-induced lupus, underscoring the importance of ERK-mediated DNMT1 expression. A polymorphism in the gene for methyl CpG-binding protein 2 (MECP2) – a methyl-binding domain containing protein that binds methylated DNA and induces transcriptional repression by the recruitment of HDACs – is associated with an increased lupus susceptibility and might provide a genetic connection between DNA methylation and the over-expression of methylation-sensitive genes in lupus patients.

There is only limited knowledge about SLE and histone modifications, but T-cells from lupus patients exhibit a global H3 and H4 hypo-acetylation and the degree of H3 acetylation correlates negatively with an increased disease activity (Pan and Sawalha, 2009).

Another disease that is very likely accompanied by chronic inflammation and which is clearly associated to aberrant DNA methylation is atherosclerosis. In advanced human atherosclerosis, global DNA hypomethylation has been found in vascular tissue as well as in peripheral blood lymphocytes. This hypomethylation might be a result of hyperhomocysteinaemia – homocysteine inhibits the conversion of methionine to S-adenosyl methionine which is the methyl donor in DNA methylation – a well-known risk factor for atherosclerosis. However, even if advanced atherosclerosis is accompanied by DNA hypomethylation, DNA hypermethylation has been described in the early stage of atherosclerosis. Additionally, the hypermethylation of specific genes like the oestrogen receptor gene in cardiovascular tissue is associated with an increased risk for atherosclerosis (Turunen *et al.*, 2009).

3.5 MicroRNA: biogenesis, function and role in regulating adaptive immunity

Relatively recently, a new class of endogenously expressed regulatory molecules has been discovered. This family of small non-coding RNAs comprises microRNAs (miRNAs), small interfering RNAs (siRNAs), transacting siRNAs (tasiRNAs), small scan RNAs (scnRNAs), repeat associated siRNAs (rasiRNAs) and Piwi-interacting RNAs (piRNAs). These molecules are highly conserved throughout evolution and exist in both animals and plants. Although poorly understood, all species may have the capacity for epigenetic regulation via different mechanisms (Chu and Rana, 2007). miRNAs have gained great attention as key regulatory molecules in diverse cellular processes and represent an emerging field of study in immunology.

The majority of miRNA loci, approximately 70%, are located in introns and/or exons, while approximately 30% are located in intergenic regions and are transcribed in parallel with other transcripts (Rodríguez *et al.*, 2004). The primary function of these 20–22 nucleotide (nt) long miRNAs is the regulation of about 30% of all messengerRNAs (mRNA) (Carthew and Sontheimer, 2009), by partially hybridizing to the 3'-untranslated region (3'-UTR) of their target and either inducing degradation of the target or repressing the process of protein synthesis. miRNAs are found in all tissues, while each cell type expresses a multitude of different miRNAs, and the expression levels of these miRNAs change from cell type to cell type. Each miRNA species further displays a unique spatiotemporal expression pattern. This gene-regulatory system provides a more conditional and flexible option when mRNA expression has to be adapted to different levels in different cell types, thereby shaping the cellular phenotype during delicate processes like development, differentiation, activation, cell proliferation and apoptosis

(Lagos-Quintana *et al.*, 2002; Houbaviy *et al.*, 2003; Krichevsky *et al.*, 2003; Chen *et al.*, 2004; Sempere *et al.*, 2004). Unlike transcriptional control elements, provided by DNA or histone modifications, miRNAs regulate gene expression on the post-transcriptional level. In contrast to transcriptional control, post-transcriptional control mechanisms are thought to be more responsive in terms of speed and reversibility, thereby allowing the cell to adapt faster and more adequately to a changed micro-environment.

So far, ~700 miRNAs have been identified in the human genome, while one miRNA is predicted to have several hundred targets. The existence of more than one miRNA complementarity sequence in 3'-UTRs of a single mRNA implies that the activity of multiple miRNAs regulates gene expression. With the potential to target a multitude of different mRNAs, individual miRNAs are able to coordinate and/or fine-tune the level of protein expression of a cell. Protein products of genes, which should not be expressed in a certain cell type, can therefore be dampened to negligible levels. For other genes, regulation by miRNAs provides a fine-tuning mechanism that allows for customized expression. Based on these considerations, miRNA levels and activity are tightly and dynamically regulated, particularly during rapid developmental transitions or changes in cellular environment. Yet, due to their genomic location, the expression of miRNAs can be tremendously affected by the cellular state, like stress or activation, caused by environmental factors, including bacterial and viral components (Taganov *et al.*, 2006; O'Connell *et al.*, 2007; Tili *et al.*, 2007). Dysregulation of miRNA expression is associated with augmented pro-teins levels in several human pathologies, including cancer, autoimmunity and allergy.

3.5.1 Biogenesis and function

The biogenesis of mature miRNAs from long primary transcripts is achieved by a series of endonucleolytic steps (Fig. 3.2). First, primary miRNA transcripts (pri-miRNAs), ranging from hundreds to thousands of nt in length, are transcribed from the genome, most commonly by RNA polymerase II. Like other RNA polymerase II transcripts, pri-miRNAs carry a 5' 7-methylguanosine cap and 3' poly (A) tail (Cai *et al.*, 2004; Lee *et al.*, 2004). Pri-miRNAs are recognized and processed by the nuclear RNase III enzyme Drosha and its partner protein DiGeorge syndrome critical region gene 8 (DGCR8) to form precursor miRNA (pre-miRNA) molecules of ~70 nt length with the typical stem-loop hairpin structure. An alternative, Drosha-independent pathway to generate a subset of pre-miRNA hairpins from introns is provided by the combined actions of the spliceosome and the lariat-debranching (LDBR) enzyme (Ruby *et al.*, 2007). Pre-miRNAs are exported from the nucleus by the RanGTP-dependent dsRNA-binding protein exportin 5.

On reaching the cytoplasm, the RNase III enzyme Dicer further processes the pre-miRNAs, removing the terminal loop and forming a miRNA-duplex of ~22 nt length (Lee *et al.*, 2003; Denli *et al.*, 2004). The miRNA-duplex consists of the functional miRNA strand and the passenger (*) strand (miRNA/miRNA*). The functional miRNA (mature miRNA) strand is incorporated into the miRNA-

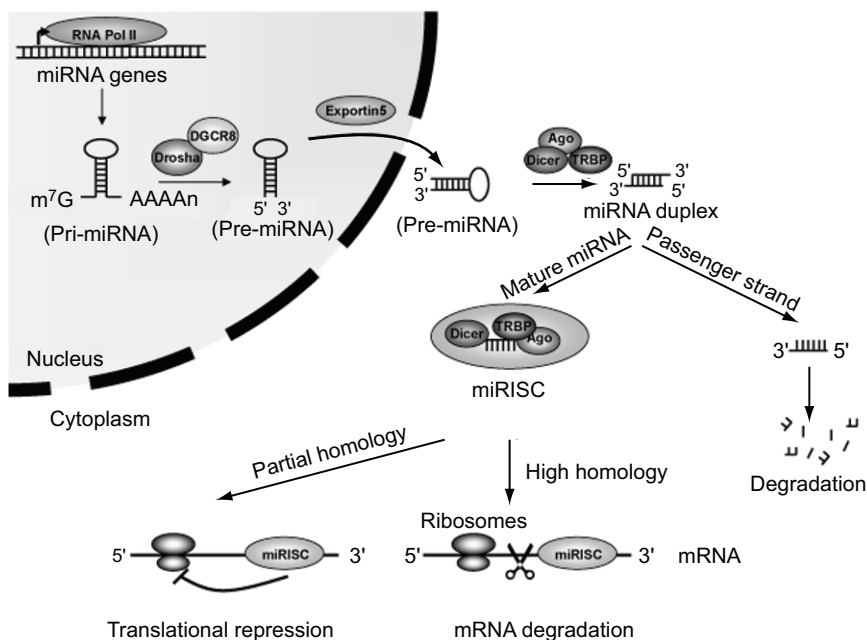


Fig. 3.2 Biogenesis and function of microRNAs.

induced silencing complex (miRISC), comprising Dicer, transactivating response RNA-binding protein (TRBP) and the nuclease argonaute (Ago). At the same time, the passenger strand is degraded.

Once incorporated in the cytoplasmic miRISC complex, the mature miRNA guides the miRISC to, and partially base pairs with, the target 3'-UTRs. In contrast to small interfering RNAs (siRNA), which require full sequence complementarity, base pairing of the seed sequence, comprising the 5 nt at positions 2–7 of the miRNA, is sufficient for hybridization to the target sequence. If the miRNA has sufficient complementarity to the mRNA, cleavage of the mRNA by Ago occurs. If the miRNA does not have sufficient complementarity, the translation of the bound mRNA is blocked. Both processes finally result in reduced expression of the corresponding protein

3.5.2 miRNAs regulating T-cell function

During inflammation, a wide variety of genes participate to ensure proper host defence and, at the same time, prevent consequences of dysregulated gene expression. During an acute inflammatory response, a functional role for several miRNAs has been implicated in the coordination of innate immune cells, e.g. miR-155 is induced by Toll-like receptor signalling and regulates TNF- α production by macrophages. In addition to regulating innate immune responses, miRNAs have been shown to play an important role in modulating adaptive immune responses as well.

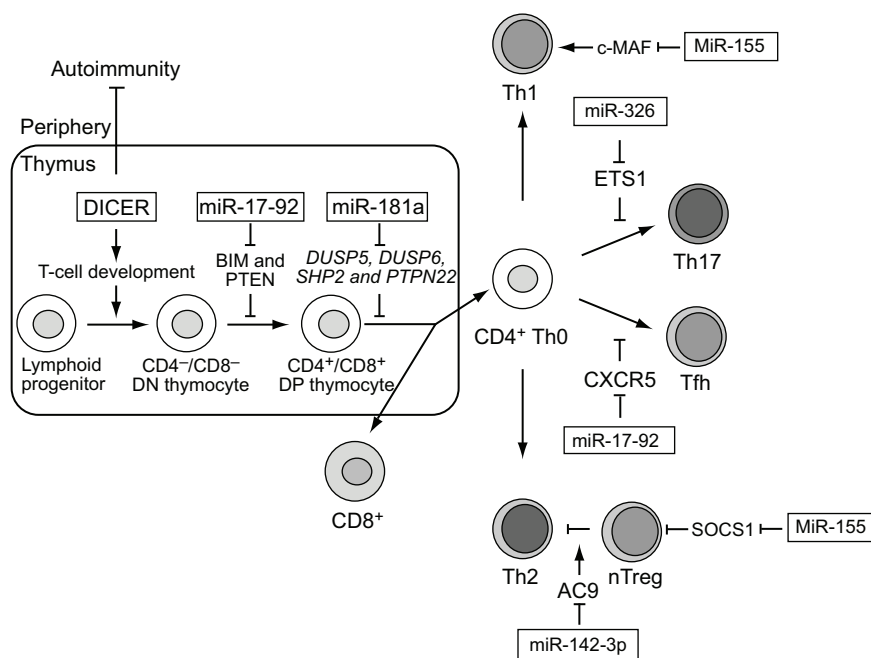


Fig. 3.3 MicroRNAs in T-cell biology. T-cell developmental processes, differentiation into distinct Th-cell subsets and their function are controlled by the RNAi pathway and the activity of microRNAs. DN, double negative; DP, double positive; miR, microRNA.

The development of T-cells in the thymus as well as their activation in the periphery is controlled by complex signalling networks, which themselves are subject to regulation by miRNAs (Fig. 3.3). Using expression profiling, a broad range of miRNA species has been identified in T-cells. The miRNA expression patterns were found to vary, either in quantity or content, between different T-cell subsets and developmental stages (Neilson *et al.*, 2007). The requirement of gene regulation in the process of mature T-cell development by miRNAs has been demonstrated by studying mice bearing a T-cell-specific deletion of Dicer. As a consequence of this deletion, knockout mice displayed lower total T-cell numbers compared to wild-type mice. Two specific miRNAs, the miR17–92 cluster and miR-181a, have been implicated in T-cell development. The miR17-92 is expressed during the double negative 2 stage of thymopoiesis. By targeting mRNAs encoding for pro-apoptotic proteins, including BCL-2 interacting mediator of cell death (BIM) and phosphatase and tensin homologue (PTEN), this cluster is thought to increase T-cell survival during development (Xiao *et al.*, 2008). This cluster has also been implicated in the development and function of follicular helper (Tfh) cells, which are specialized cells dedicated to supporting B cells in germinal centres and facilitating class switching and affinity maturation.

A pivotal role for miR-181a has been documented in thymic development. During this period, positive and negative selection of thymocytes ensures proper

development of immunity and tolerance and is influenced by the strength of TCR-signalling. Compared to differentiated Th1 and Th2 cells, the expression of miR-181a is increased during early T-cell development and targets mRNAs encoding for a group of protein phosphatases, including dual-specificity protein phosphatase 5 (*DUSP5*), *DUSP6*, SH2-domain-containing protein tyrosine phosphatase 2 (*SHP2*; also known as *PTPN11*) and protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*). By directly targeting these phosphatases, miR-181a causes elevated steady-state levels of phosphorylated intermediates and reduces the T-cell receptor signalling threshold, and thus augmenting sensitivity of immature T-cells to antigens (Li *et al.*, 2007).

More recent data suggest a role for miRNAs in lineage commitment of effector T-helper cell subsets, a process which is strongly dependent on complex signalling networks and the expression of distinct transcription factors. Additionally to cytokine and transcription factor feedback loops, T-helper cell differentiation and lineage commitment are controlled by epigenetic mechanisms of gene activation and silencing (Ansel *et al.*, 2003, 2004). The first hints that the RNAi pathway may also participate in the epigenetic silencing of relevant genes during helper T-cell lineage commitment were obtained from studies performed in Dicer-deficient T-cells. While differentiation of CD4⁺ T-cells into Th1 and Th2 lineages can occur in the absence of Dicer, stable commitment of differentiated subsets seems to be strongly dependent on the RNAi pathway. Dicer-deficient Th2 cells are unable to silence IFN- γ production adequately, and can therefore be reprogrammed into Th1 cells (Muljo *et al.*, 2005). Although a specific miRNA has not been identified yet, these data imply that the RNAi pathway is important in controlling aberrant Th1 responses.

Th2-differentiation seems to be tightly controlled by miRNA-155. In mice carrying a null mutation in the miR-155 gene, T-cells are intrinsically biased to produce higher amounts of Th2 cytokines IL-4, IL-5 and IL-10. Further studies revealed that this was due to higher expression of the Th2-promoting transcription factor c-Maf in knockout mice. In contrast, IFN- γ production by Th1 cells was not augmented by miR-155 knockout (Rodriguez *et al.*, 2007; Thai *et al.*, 2007). This implies that the expression and activity of miR-155 critically regulates Th2 immune responses.

Differentiation of T-helper cells into Th17 cells is induced by the induction of RoR γ t expression in T-cells and negatively regulated by ETS1. miR-326 might induce Th17 cells by directly targeting ETS1, a transcription factor identified as a negative regulator of Th17 differentiation (Moisan *et al.*, 2007).

A crucial role for miRNAs in Treg cell biology was shown by targeted deletion of Dicer and Drosha in Foxp3⁺ Treg cells. Mice bearing these deletions show impaired development or function of Treg cells, causing lethal autoimmune inflammatory disease (Chong *et al.*, 2008; Liston *et al.*, 2008; Zhou *et al.*, 2008). Specifically, a functional role for miR-155 and miR-142-3p in Treg cells was demonstrated. miR-155 regulates survival and homeostasis of Treg cells by directly targeting suppressor of cytokine signalling 1 (SOCS1) (Kohlhaas *et al.*, 2009; Lu *et al.*, 2009). The regulatory activity of Treg cells, suppressing T-helper

cell activity, is exerted by transferring cyclic AMP (cAMP) to responder T-cells. High levels of cAMP are maintained by adenylyl cyclase (AC) activity. AC9 expression is regulated by miR-142-3p, which itself can be directly blocked by Foxp3. By dampening the expression of miR-142-3p expression, Treg cells sustain their suppressive activity and ensure immune tolerance (Huang *et al.*, 2009).

As discussed above, miRNAs adjust the expression of various regulatory proteins needed for normal development and function of the immune system. Yet, an imbalance of miRNA expression is sufficient to cause disease. The observation that a single miRNA can have several affects on different cell types hints toward a unique function of miRNAs in distinct cell subsets, like transcription factors. Furthermore, a regulatory loop between miRNAs and transcription factors exists, and this might help in establishing stable cellular identities during development. Therefore, proper regulation of miRNA expression seems to be critical to prevent disease pathology.

3.6 Conclusion and future trends

Chronicity of inflammation is, to a large extent, controlled by a profound dysregulation within the adaptive immune response. This dysregulation is characterized by the development and activation of certain T-helper cell subsets. Whereas allergic diseases (asthma, eczema, food allergy and allergic rhinoconjunctivitis) are controlled by Th2 immune cells, many autoimmune disorders of the gut, joints, pancreas and brain are dominated by Th1 and Th17 subsets. Furthermore, in many of these diseases, diminished activity or reduced frequency of regulatory T-cells has been described. Chronic inflammatory conditions are considered as the result of complex gene–environment interactions. In this regard, the question arises of how environmental factors such as nutritional components, microbes, pollutants and others may influence the gene expression profile in the host. The discovery of epigenetic mechanisms now provides the platform for a better understanding of the operational network of gene–environment interaction on the molecular and cellular level. New studies need to be designed in order to identify environmental components in more detail which are able to modify epigenetic marks.

Another level of investigation should be directed towards the identification of the underlying mechanisms. Results from such studies might then lead to the design of novel strategies, able to interfere in a preventive/protective or therapeutic fashion in this regard. In terms of timing, it has now been well established that early life comprising pre- and postnatal periods is an important window of opportunity where the host exhibits a high degree of susceptibility for programming protective or, in contrast, disease-related immune responses. This ‘window’ also offers the chance for novel approaches aimed to protect against later chronic inflammatory disease. Therefore, there is the realistic chance of counteracting the prominent trend of increasing chronic inflammatory conditions worldwide.

3.7 References

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104 Diet, immunity and inflammation

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4

Chronic low-grade inflammation

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Abstract: Obesity is associated with chronic low-grade inflammation which results in insulin resistance, type 2 diabetes, vascular disease, chronic renal failure, several cancers and endocrine and behavioural abnormalities. The enlarged white adipocytes of the obese are now considered to be the initiators of chronic low-grade inflammation. Indeed, the gradual accumulation of triglycerides within adipocytes shifts their phenotype towards a pro-inflammatory one and at the same time accelerates their apoptosis. The hyperplastic white trunkal adipocytes of obesity produce large amounts of pro-inflammatory cytokines, acute-phase proteins and chemokines which attract circulating monocytes into adipose tissue and induce their activation to macrophages which intensify further and expand the adipose tissue inflammation.

Keywords: chronic low-grade inflammation (CLGI), obesity, adipose tissue, insulin resistance, T cells.

4.1 Introduction

Obesity is associated with chronic low-grade inflammation (CLGI). Since the 1990s, a tremendous amount of published research has expanded our understanding of the pathophysiological mechanisms involved in generation of CLGI in obesity and revealed its central role in the development of insulin resistance and vascular endothelium damage. Indeed, CLGI has emerged as a new pathophysiological entity in its own right. CLGI is of central importance in the development of type 2 diabetes, coronary and cerebral vascular disease, chronic renal failure, several cancers and endocrine and behavioural abnormalities. As a result, it is now a major therapeutic target. CLGI should be diagnosed and treated early in order to prevent its consequences. The source of CLGI in obesity appears to be the

expanded trunkal adipose tissue. The inflammation within adipose tissue subsequently spreads to the rest of the body. It is fascinating to recall that up to a couple of decades ago, adipose tissue was considered a tissue of minimal physiological importance, at best a depot of excess energy storage in the form of triglycerides. However, this view has changed dramatically and adipose tissue biology has emerged as a most active area of scientific research.

4.2 Obesity and the development of chronic low-grade inflammation (CLGI)

As mentioned above, in obesity the expanded trunkal adipose tissue initiates a cascade of events causing a local inflammatory response which very rapidly spreads systemically. Several factors have been implicated in the generation of the inflammatory response in obesity. The most important appear to be the following:

- Tissue hypoxia plays a central role.¹ Adipose tissue hypoxia results from the rapid expansion of adipose tissue in obesity which greatly surpasses the perfusion capacity of the existing vasculature, since its growth lags behind expanded adipose tissue demand for oxygen. The significance of tissue hypoxia is now beyond any doubt.²
- An increasing number of authors believe that the most important determinant for the development of inflammation within the adipose tissue is the increase in the size of adipocytes.^{3,4} The accumulation of more and more triglycerides within each adipocyte causes their gradual enlargement which appears to cause a shift of their phenotype towards a pro-inflammatory one (see Fig. 4.1).
- In addition, the enlarged adipocytes undergo apoptosis accelerating the local inflammatory reaction. It is now believed that the cut-off in adipocyte size for apoptosis is lower in trunkal adipocytes as compared to subcutaneous adipocytes⁵ making the former much more dangerous. Furthermore, during obesity the ratio of trunkal to subcutaneous adipocytes favours the former resulting in a large number of adipocytes undergoing apoptosis and thereby inducing innate immunity mechanisms.⁶
- Finally, changes in the ratio of white to brown adipocytes within adipose tissue appear to play a role in CLGI in obesity.⁷

Obesity also appears to enhance the usually mild postprandial inflammatory reaction.⁸ This transient reaction, which follows every meal, depends on the components of the ingested meal and peaks four to eight hours postprandially.⁹ Unfortunately, it appears to be greatly accentuated in obesity, its intensity correlating with the size of the adipocytes.¹⁰

4.2.1 Insulin resistance caused by chronic low-grade inflammation

The mechanism by which CLGI causes insulin resistance is complex but the principal steps are as follows:

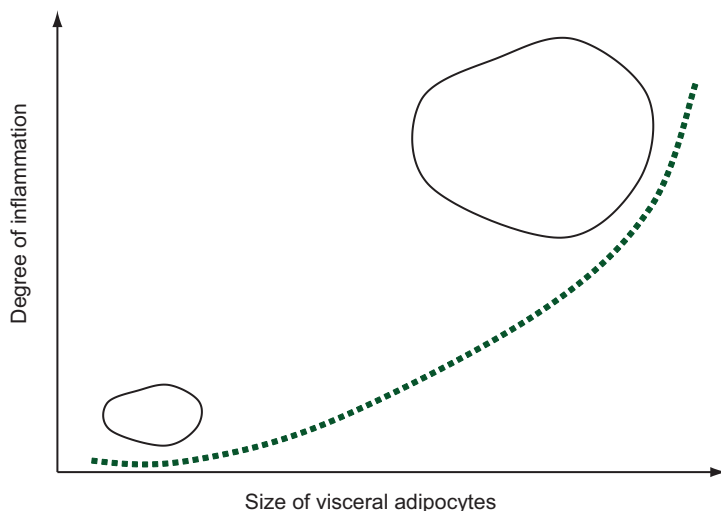


Fig. 4.1 Hypothetical relationship between adipocyte size and inflammatory phenotype. It appears that the accumulation of esterified fatty acids in triglycerides within adipocytes triggers their pro-inflammatory phenotype, the degree of which is directly related to cell size.

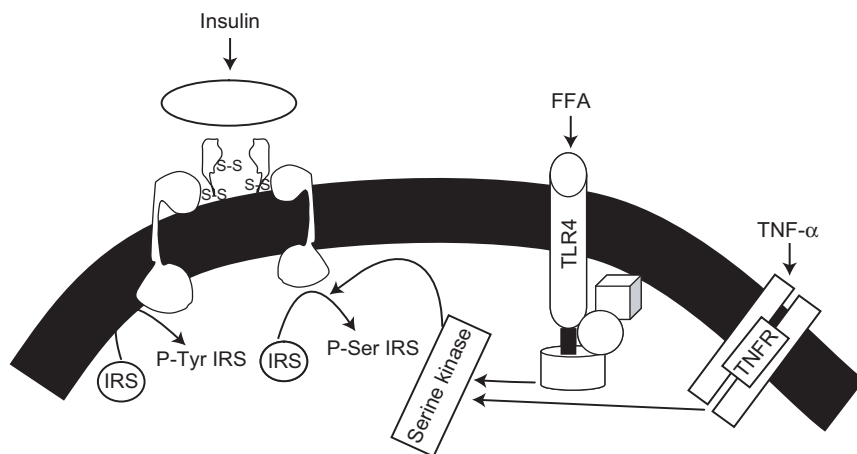


Fig. 4.2 Chronic low-grade inflammation induces insulin resistance. Certain lipids (e.g. some free fatty acids: FFA) and pro-inflammatory cytokines (e.g. TNF) result in phosphorylation of insulin receptor substrate (IRS) on specific Ser residues decreasing IRS availability for phosphorylation (on specific Tyr residues) by the insulin receptor. TLR, toll-like receptor; TNFR, TNF receptor.

1. Pro-inflammatory cytokines activate kinases I κ B kinase beta (I κ K- β) and C-jun N-terminal kinase 1 (JNK), both of which phosphorylate the serine 307 residue of insulin receptor substrate 1 (IRS-1), thus blocking tyrosine phosphorylation of IRS-1, the normal second step following activation of insulin receptor by insulin (see Fig. 4.2).¹¹
2. The pro-inflammatory cytokines enhance the activity of two proteins, suppressor of cytokine signaling (SOCS) 1 and 3, preventing further tyrosine phosphorylation of IRS by the activated insulin receptor.^{12,13} The expression of SOCS1 and 3 is induced by several cytokines and they act as negative regulators of cytokine signaling, i.e. they act as brakes of an ongoing inflammatory event.
3. Several pro-inflammatory cytokines cause insulin resistance via activation of p38 mitogen-activated protein kinase (MAPK) which interferes with the IRS-phosphatidylinositol 3-kinase/Akt pathway.¹⁴

4.3 Adipose tissue as an immune organ

In addition to being the largest metabolic and endocrine organ, adipose tissue is also a highly active immune organ. It produces almost 100 hormones, immune mediators, growth factors and chemoattractant proteins.¹⁵ Via these substances, adipose tissue affects innate and adaptive immunity. The ‘fat body’ concept addresses the question of why an obviously metabolic organ possesses so many immune characteristics and local (resident) immune cells. Briefly, the hypothesis behind this concept states that the ‘metabolic cells’ responsible for handling of energy (e.g. hepatocytes and adipocytes) are in close proximity with immune cells as a result of an evolutionary drive for an interaction.¹⁶ Indeed, from an evolutionary point of view, an intense interaction between metabolic and immune cells gives an evolutionary advantage to species in that they can coordinate their metabolism in an efficient manner handling energy and at the same time combating invading micro-organisms. For instance, in *Drosophila melanogaster*, energy-handling cells (hepatocytes and adipocytes) and the hematopoietic/immune cells all reside in a single organ, the fat body, an organ central in the coordination of the three systems.¹⁷

In higher organisms, the close association between immune and energy-handling cells has been retained although each particular function has been assigned to specific tissues and organs. However, it should be noted here that immune and metabolic cells retain their close interaction and common characteristics. For example, adipocytes, immune cells and liver cells all express Toll-like receptors (TLRs) on their surface and respond to the appropriate ligands using more or less the same signaling pathways. The structure of these organs is also common. Adipocytes are in close proximity to tissue macrophages within adipose tissue and hepatocytes are surrounded by Kupffer cells which are specialized macrophages located in the liver lining the walls of the sinusoids that form part of the local reticuloendothelial system. Such structures support functional cross-talk

between these cell types. An efficient response to an invading pathogen requires intensification of energy expenditure via insulin signalling. Indeed, immune cells produce factors that affect adipocytes while the latter produce factors which affect immune cells. Thus, adipocyte-derived leptin directly affects immune cells, modulating their responses to pathogens.¹⁸

Adipocytes also produce adiponectin, an adipokine with multiple functions including direct effects on immune cells.¹⁹ In lean individuals, in whom adipocytes are not overloaded with lipids, adipocytes produce low amounts of leptin while they produce increased amounts of adiponectin. Adiponectin attenuates immune responses primarily by suppressing macrophage activation, thus maintaining immune homeostasis by avoiding hyperactivation of macrophages in response to pathogens or endogenous stimuli such as apoptotic cells or microbiota in the intestinal tract.

4.4 White and brown adipose tissue and CLGI

Adipose tissue is composed of several types of cells including mature adipocytes, pre-adipocytes as well as stroma cells including histiocytes, fibroblasts, vascular endothelial cells, macrophages and T-regulatory lymphocytes. Classically, two types of adipose tissue are recognized: the white adipose tissue (WAT) and the brown adipose tissue (BAT) which we now know persists in the adult and plays a crucial role in energy balance.

4.4.1 Characteristics of white adipose tissue (WAT)

In adults, WAT is localized either subcutaneously or within the trunk (pericardium, on the omentum, retroperitoneally and around the viscera). It should be noted here that current research data indicate that the white adipocytes in the subcutaneous adipose tissue differ in several aspects from those within the trunk. The most important difference appears to be their cut-off size for triggering apoptosis which leads to the inflammatory response. Indeed, trunkal white adipocytes undergo apoptosis at a much smaller size, while subcutaneous adipocytes are more resistant, being able to store energy and enlarge without turning into an apoptotic mode.²⁰ Similarly, the increase in size of white adipocytes in the trunk in obesity changes their phenotype into a pro-inflammatory one while the subcutaneous white adipocytes are more resistant retaining their non-inflammatory phenotype.²¹

4.4.2 Characteristics of brown adipose tissue (BAT)

We now know that BAT persists in adults, being detectable in the cervical, supraclavicular, axillary and paravertebral regions as documented by positron emission tomography (PET) scans (showing areas of metabolically active fat) and

Table 4.1 Comparison of the characteristics of white and brown adipocytes

Characteristic	White adipocytes	Brown adipocytes
Cytosolic fat	One large fat lobule (unilocular cells)	Multiple small fat droplets (multilocular cells)
Mitochondria	Few and scattered	Large numbers with characteristic large cristae
Role in energy handling	Storage energy as triglycerides	Expend energy as heat
Uncoupling proteins (UCP)	UCP2	UCP1 (thermogenin), UCP2
Adipokines produced	Many in high concentrations	Few in low concentrations
Cytokines produced	Mainly pro-inflammatory	Mainly anti-inflammatory

CT scans (showing areas of fat irrespective of their metabolic activity).²² Even more spectacular is the demonstration that white adipocytes can be induced to change into a brown phenotype i.e., transdifferentiate into BAT adipocytes.²³

4.4.3 Differences between WAT and BAT

There are several differences between WAT and BAT including vasculature (it is denser in BAT) and handling of energy. Indeed, white adipocytes store energy while brown adipocytes expend it. Histologically, white adipocytes contain a single large depot of stored triglycerides (unilocular) while brown adipocytes are characterized by a fragmented depot of triglycerides i.e., multiple small fat vesicles (multilocular) on their way to enter mitochondria and be converted ultimately to heat. Indeed, brown adipocytes are extremely rich in mitochondria which contain large amounts of the uncoupling protein-1 (UCP-1) which is located within the inner mitochondrial membrane and allows protons to escape mitochondria, thus preventing them from producing high-energy compounds like adenosine triphosphate (ATP) instead losing energy as heat. In summary, the principal function of white adipocytes is to store energy (in the form of triglycerides) while that of brown adipocytes is to expend energy (in the form of heat). In addition to the way they handle energy, white adipocytes differ from brown adipocytes in several other aspects, including expression of several developmental genes, response to insulin, response to adrenergic innervation and, more importantly, in their immune properties (see Table 4.1).⁷ Briefly, white adipocytes have a tendency to develop a pro-inflammatory phenotype while brown adipocytes develop an anti-inflammatory one.⁶

4.5 Adipocytes as immune cells

The first indication that adipocytes produce immune mediators was by Hotamisligil *et al.* in, 1993.²⁴ They demonstrated that visceral fat adipocytes express the tumour necrosis factor (TNF)- α gene, its expression increasing in obese and insulin-resistant persons. We now know that adipocytes are close relatives of macrophages,

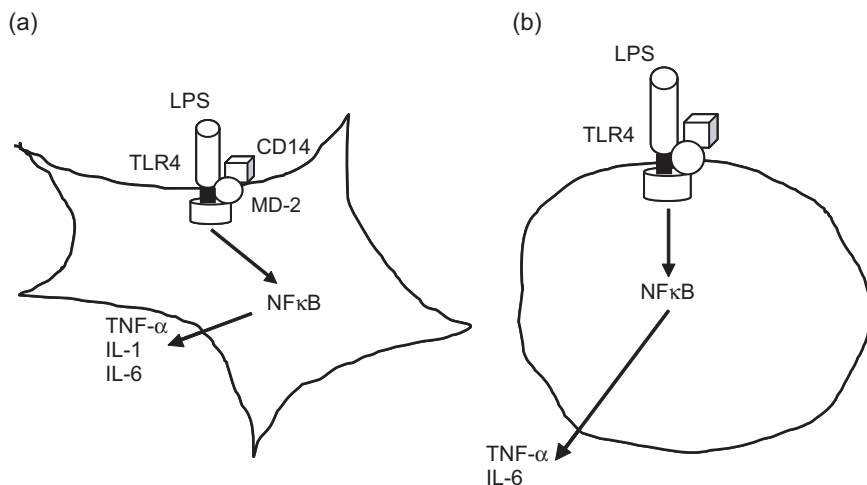


Fig. 4.3 The TLR4-NFκB-pro-inflammatory cytokines cascade is common in macrophages (a) and large trunkal white adipocytes (b). LPS, lipopolysaccharide.

sharing several common characteristics (see Fig. 4.3). Indeed, the TLR4–nuclear factor kappa B (NFκB)–pro-inflammatory cytokines cascade is common in both cell types but, in small lean adipocytes, does not appear to be activated. Obesity enhances the inflammatory phenotype of adipocytes most probably via their size enhancement. Several hypothetical models try to explain the pathway via which this is achieved.

The first model holds that in obesity the enlarged adipocytes secrete more free fatty acids which induce nearby located macrophages to express the TNF-α which is released by macrophages, binds to its receptors in the nearby adipocytes and triggers their pro-inflammatory phenotype (see Fig. 4.4).²⁵ The adipocytes thus activated produce chemoattractant proteins which attract more and more macrophages into the adipose tissue via transendothelial migration of monocytes into adipose tissue. The most important chemoattractant protein is the monocyte chemoattractant protein-1 (MCP-1) or C-C motif chemokine ligand-2 (CCL2). Indeed, high levels of the *CCL2* (MCP-1) transcript are present in adipocytes from obese Pima Indians.²⁶ It should be noted that not only the number but also the type of macrophages changes in the adipose tissue in obesity. Indeed, obesity induces a phenotypic switch in the type of macrophages within the adipose tissue from the M2 phenotype which is anti-inflammatory, i.e. these macrophages produce mainly interleukin (IL)-10 and IL-1ra to the M1 phenotype.²⁷ These macrophages produce mainly TNF-α, IL-1 and IL-6. Thus, in normal weight individuals the ratio of M1/M2 is less than 1 while in the obese it is more than 1.²⁷

The second model is based on the STAMP2 hypothesis. STAMP2 (six-transmembrane protein of prostate) is a membrane receptor expressed in lean adipocytes and blocks their TLR4 and cytokine receptors cascade.²⁸ The endogenous ligand for this receptor is not yet known. In obesity, the expression of

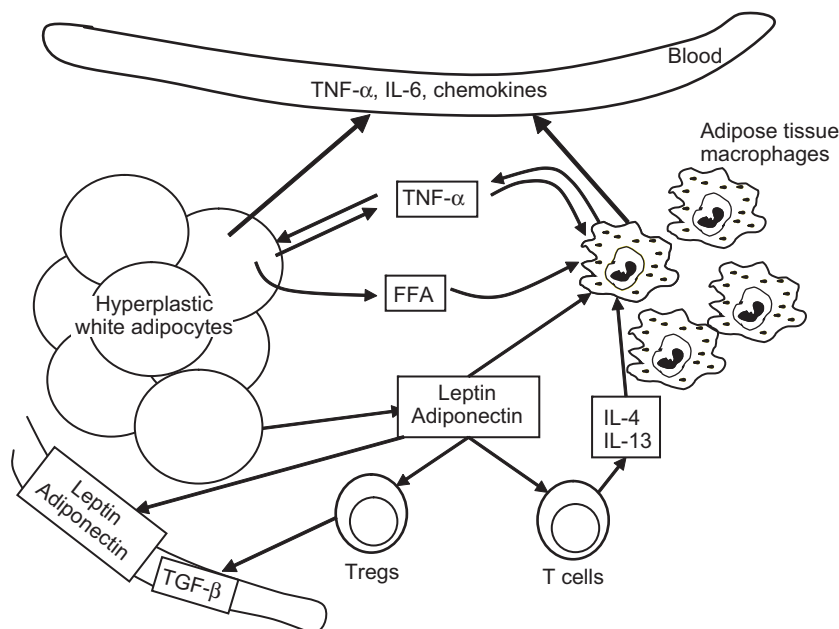


Fig. 4.4 Cellular interactions in metabolic inflammation. Large trunkal white adipocytes secrete free fatty acids (FFA) which induce nearby macrophages to produce TNF- α which binds to its receptors on nearby adipocytes and triggers their pro-inflammatory phenotype. T cells are influenced by adipocyte-derived factors and, in turn, T cells influence macrophage responses.

STAMP2 is reduced;²⁹ thus the brake of the inflammatory cascade in adipocytes is released.

4.5.1 Large trunkal white adipocytes and CLGI

An inflamed adipose tissue is now considered a major histological characteristic of trunkal fat in obesity and the initiator of CLGI. The inflamed fat is characterized by the presence of an elevated number of macrophages within it and an increase of apoptotic adipocytes, each surrounded by macrophages.³⁰ The hyperplastic white trunkal adipocytes of obesity produce pro-inflammatory cytokines, acute-phase proteins and chemokines (CCL2/MCP-1) which attract more monocytes/macrophages into the adipose tissue.³¹ This vicious cycle results in the development of inflammation within the visceral adipose tissue as well as systemically. This intra-adipose tissue paracrine cross-talk between hyperplastic adipocytes and local macrophages is intense and is based on the common characteristics of these two cell populations. Indeed, as has been already mentioned, adipocytes and macrophages share several characteristics, including the presence of TLR4 receptors (response to lipopolysaccharides, LPS) and the ability to synthesize inflammatory cytokines (Fig. 4.3).

4.5.2 Obesity and white adipocyte apoptosis

Excessive loading of adipocytes with triglycerides initiates their apoptosis. Apoptotic adipocytes trigger a local inflammatory response and recruitment of macrophages to adipose tissue.³¹ Even though normally only a small percentage of cells in the adipose tissue (5–10%) are macrophages, in obese individuals the adipose tissue may contain macrophages accounting for up to 50% of the total cells present.³² This local inflammation switches macrophages to the M1 type of response, resulting in elevation of pro-inflammatory cytokine production.

4.6 Impact on inflammation of other cells from adipose tissue

Apart from the adipocytes, other cells within the adipose tissue participate in the generation of metabolic inflammation. Generally, we can divide the adipose tissue cells into two categories: the ones that induce metabolic inflammation, which includes white adipocytes, adipose tissue resident macrophages of the M1 type and vascular endothelial cells; and cells that suppress metabolic inflammation which includes brown adipocytes, adipose tissue resident macrophages of the M2 type and adipose tissue T helper cells (see Table 4.2).

4.6.1 Adipose tissue monocytes/macrophages and CLGI

Monocytes and macrophages are the predominant population of immune cells within the adipose tissue. Macrophages produce pro-inflammatory factors and they also phagocytose apoptotic adipocytes, the number of which increases in an exponential number in obesity. As mentioned above, it now appears that trunkal fat white adipocytes are very vulnerable to the accumulation of triglycerides within them and the cut-off size limit to trigger apoptosis is much lower compared to the subcutaneous ones. Indeed, white adipocytes appear to be very sensitive to size change. Macrophages that lie in their vicinity readily take up apoptotic bodies to prevent further tissue damage. Macrophages can exhibit enhanced phagocytic properties when they have differentiated to the alternatively activated macrophages or M2 type.³³ In the adipose tissue, macrophages have primarily M2-type properties and are therefore primed for their role as scavengers. Macrophages differentiate

Table 4.2 Involvement of adipose tissue cells in inflammation

Cells that induce metabolic inflammation

- White adipocytes
- Adipose tissue resident macrophages of the M1 type
- Vascular endothelial cells

Cells that suppress metabolic inflammation

- Brown adipocytes
 - Adipose tissue resident macrophages of the M2 type
 - Adipose tissue T helper cells
-

from monocytes after recruitment to peripheral organs and exposure to the local cytokine milieu. Such recruitment is prominent in obesity. Differentiated macrophages can be categorized into two types, based both on function and on the profile of cytokine expression. M1 macrophages produce IL-1, IL-6, TNF- α and other pro-inflammatory cytokines, resulting in neutrophil and T cell recruitment and allowing the initiation of the immune response to pathogens (Fig. 4.4). M1 macrophages are triggered primarily by pathogens that bind on pathogen-associated molecular patterns (PAMPs), such as TLRs that initiate signalling cascades activating transcription factors that promote production of pro-inflammatory cytokines and chemokines that also activate T cells triggering the adaptive immune system. T cells are stimulated by pro-inflammatory cytokines and ligands specific for their individual T cell antigen receptors (TCRs). Activated T cells proliferate and release cytokines, including interferon (IFN)- γ , IL-6, and TNF- α , augmenting the response to the pathogens by further driving differentiation and activation of M1 macrophages, recruitment of more lymphocytes and initiation of the adaptive immune response. Both T cells and M1 macrophages are associated with chronic inflammation in metabolic diseases.³³ M1 macrophages are scarce in the lean adipose tissue and the predominant macrophage phenotype found is the M2.³¹ M2 macrophages (alternatively activated macrophages, AAMs) represent a broad range of macrophage types that share some common features. M2 macrophages are those that contribute to wound healing and tissue repair responses.³⁴ M2 macrophages secrete the anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)- β . Both of these cytokines are also released by CD4⁺Foxp3⁺ regulatory T cells (Tregs) and also drive Treg differentiation. Tregs and M2 macrophages reside in the adipose tissue of lean mice and in healthy peripheral tissues of insulin-responsive animals. M2 macrophages occur via stimulation with IL-4, or IL-10 or TGF- β , or combination of these cytokines. These factors promote suppression of inflammation and contribute to phagocytosis and tissue remodelling. Recent reports have indicated that the anti-inflammatory adipokine adiponectin can also promote M2 macrophage differentiation.^{35,36} Adipocytes in lean adipose tissue produce elevated levels of adiponectin, thus supporting the generation of M2 macrophages. An additional hallmark of murine M2 macrophages is expression of arginase. Arginase competes with inducible nitric oxide synthase for the same substrate, L-arginine, and converts it to urea, thus inhibiting the generation of NO, a major pro-inflammatory molecule.³⁷ As a result, presence of M2 macrophages in the adipose tissue of lean individuals maintains a low inflammatory status.³¹ In obesity, the number and type of macrophages within the white adipose tissue change, large numbers of monocytes are recruited by the hyperplastic adipocytes from the systemic circulation; they enter the adipose tissue where they mature into active macrophages. The rapidly increasing number of macrophages within adipose tissue is accompanied by a change of their phenotype from an anti-inflammatory one (the M2 phenotype) to the pro-inflammatory one (the M1 phenotype).

It should be noted here that in obese individuals both macrophages and adipocytes produce several of the same inflammatory regulators and mediators,

including TNF- α , IL-6, matrix metalloproteinases (MMPs), peroxisome proliferator activated receptor- γ and fatty acid-binding protein-4.^{38–41} In addition, it appears that a foul and self-intensifying cycle occurs where adipocytes induce macrophages and macrophages induce further the adipocytes in their vicinity. As mentioned above, macrophages and adipocytes interact in a paracrine manner and they share several common characteristics including their ability to produce inflammatory cytokines in response to LPS via TLR4. STAMP2 suppresses the pro-inflammatory aspect of adipocytes. Indeed, STAMP2 blocks the activated inflammatory signaling pathways in adipocytes.²⁸ In obesity, the expression of STAMP2 is reduced and the adipocytes become more immunologically aggressive. In STAMP2 knockout mice, the visceral tissue is resistant to insulin action and produces pro-inflammatory cytokines. Interestingly, saturated fatty acids that induce a strong postprandial inflammatory response suppress STAMP2 gene expression in primary adipocytes in culture and in the 3T3 mouse adipocyte cell line.²⁸

4.6.2 T cells and CLGI

In recent years it has been shown that regulatory T-lymphocytes residing within adipose tissue play a role in the development of metabolic inflammation.⁴² More specifically, they represent a major mechanism for the containment of the inflammatory process within adipose tissue. Depending on the immune challenge, T helper cells can ameliorate the activity of other immune cells to generate pro-inflammatory T helper type 1 (Th1) responses through phagocyte activation or humoral Th2 responses through stimulation of B cell activity. In lean mice, resident adipose tissue macrophages have low inflammatory activity restrained by Th2 cytokines.⁴³ In obesity, the new macrophages recruited into the adipose tissue are stimulated by Th1 signals which induce them to produce pro-inflammatory cytokines.⁴⁴

Immunohistological and flow cytometry analyses have revealed several subtypes of T cells in the adipose tissue. Prominent were populations of natural killer (NK, CD49b⁺) and T cells (CD3⁺) in the close vicinity of small or larger adipocytes, in both epididymal and inguinal fat pads.⁴⁵ However, differences exist between the two anatomical locations since the subcutaneous inguinal depot contains lymph nodes, while the visceral epididymal fat pad does not. Immunohistochemical analysis revealed plasma cells (which originate from the B lymphoid lineage). Cytofluorometric analysis revealed that, in rodents, around 10% of the cells present in white adipose tissue (either epididymal or inguinal) were lymphocytes. In epididymal fat, lymphocytes display a more primitive, innate immune system-like phenotype (around 70% of NK, $\gamma\delta$ T and NKT cells among lymphocytes) whereas, in inguinal WAT, the immune system presents more adaptive characteristics (65–70% of $\alpha\beta$ T and B cells among lymphocytes).⁴⁵

These observations support that adipose tissues contain different percentages of the different types of lymphocytes. An additional subtype of T cells that is detected in lean adipose tissue is Tregs, cells that support an anti-inflammatory status in the microenvironment of the adipocytes. Tregs are generated by TGF- β and/or IL-10,

factors that are also produced by M2 macrophages, as mentioned above. The high proportion of lymphocytes together with the presence of macrophages confers a specific immune phenotype to adipose tissue.

4.7 The anti-inflammatory status of lean adipose tissue

The adipose tissue in the lean person maintains a homeostatic balance in which inflammatory signals have to be eliminated to ensure proper function of the adipocytes. For this purpose, both cell-intrinsic and cell-extrinsic mechanisms work in a coordinated manner to regulate the inflammatory response. Among cell-extrinsic mechanisms, the short half-life of pro-inflammatory cytokines limits the duration of the inflammatory response to remain at the essential time period for eliminating the insult. The anti-inflammatory cytokines TGF- β and IL-10 suppress immune cell activation and attenuate the inflammatory response. At the same time, they promote the differentiation and activation of Tregs which, in turn, suppress T-cell responses. The major anti-inflammatory adipokine adiponectin has been shown to induce IL-10 production from macrophages,⁴⁶ thus promoting Treg generation and M2 differentiation of macrophages. After release of antimicrobial molecules, macrophages clear dead cells by phagocytosis to prevent tissue necrosis. By removing dead cells, macrophages eliminate a pro-inflammatory stimulus. At the cell-intrinsic level, cells express molecules that control the magnitude of their response to noxious stimuli. Macrophages express IL-1 receptor-associated kinase (IRAK)-M, an inactive homologue of IRAK-kinases that suppresses TLR signaling.⁴⁷ Recent data suggest that adiponectin promotes IRAK-M expression,⁴⁸ thus maintaining an anti-inflammatory environment within the adipose tissue.

4.8 Laboratory evaluation of CLGI

The magnitude of metabolic inflammation is best assessed in the fasting state. Acute phase inflammatory proteins, pro-inflammatory cytokines and chemoattractant proteins are the most frequently used. High sensitivity C-reactive protein (hs-CRP) is the gold standard and is considered the best marker to be measured for clinical purposes. hsCRP <1 mg/L suggests no active CLGI; between 1 and 3 mg/L suggests clinically significant CLGI; between 3 and 10 mg/L suggests a dangerously high CLGI; and >10 mg/L suggests acute inflammation not related to CLGI. Other acute-phase proteins can also be measured to assess metabolic inflammation and include the haptoglobin, serum amyloid A (SAA) and fibrinogen. Cytokines that can be measured include IL-1, IL-1ra, IL-6, IL-8, IL-18, IL-10 and TNF- α . In addition, adhesion and remodelling molecules of extracellular matrix, chemokines and complement factors can be measured. Low caloric diets for several weeks or a small reduction in body weight can ameliorate CLGI. Several studies have shown that a decrease in food intake suppresses CLGI as indicated by a decrease of the circulating pro-inflammatory markers and a parallel

increase of the anti-inflammatory ones.^{49–55} This effect has been documented for hsCRP, IL-6, IL-18, IL-1-ra, PAI-1, SAA and MMP-9.

It is now clear that signaling molecules involved in CGLI can also be used as potential markers. In our research protocols we use IRAK-M and SOCS3. These are kinases that are involved in innate immunity by inhibiting TLR signalling. IRAK-M expression is limited to monocytes and macrophages and thus more specific than other members of this family.⁵⁶ Recent data indicate that adiponectin induces the expression of IRAK-M in mice, while adiponectin levels seem to be negatively associated to both IRAK-M and IL-6.⁴⁸ SOCS3 expression strongly correlates to IL-6 in obese insulin-resistant non-diabetic individuals.^{12,57} SOCS3 is also upregulated by TNF- α , insulin and leptin.^{58,59} Multiple lines of evidence suggest that those cytokines and several hormones upregulate the expression SOCS3 in adipocytes.^{57,58}

Finally, nutrigenomics is a new application that aims to identify molecular markers of diet-related diseases using peripheral blood mononuclear cells as a cell model system. RNA-based microarray chips are now in research use in adipose tissue biopsies.⁶⁰

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120 Diet, immunity and inflammation

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Part II

Micronutrients, immunity and inflammation

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5

The role of zinc in immunity and inflammation

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Abstract: Zinc plays a diverse role in human health. It is an essential trace element for all life forms due to its role in cell proliferation, transcription and enzyme activity. Since the immune system is the organ system with the highest proliferation rate, zinc is crucial for maintaining an intact immune system and affects virtually all of its components. The chapter first gives an overview of zinc nutrition and the role of zinc in human health. The role of zinc in the modulation of cellular processes is discussed on the basis of the altered molecular mechanisms. The influence of zinc on the immune system is reported in more detail. Finally therapeutic uses and future options of zinc supplementation are critically analysed.

Key words: zinc, immune system, inflammation, trace element, cell signalling.

5.1 Introduction

Zinc is an element that plays an important role in human health. It is not only crucial for maintaining an intact immune system, but is also of vital significance in many other organ systems and processes in the human body. The influence of zinc can be readily seen in highly proliferating organ systems like the immune system, the skin and the male reproductive system.

The first observations in humans concerning zinc were made in the 1960s by Prasad *et al.*, who described a zinc deficiency syndrome in Iranian children. The children suffered from anaemia, were physically and mentally retarded, and they showed skin alterations, hypogonadism and hepatosplenomegaly. They usually died before the age of 25 years as they were extremely susceptible to bacterial,

fungal and parasitic infections, due to an immune deficiency (Prasad *et al.*, 1963). All symptoms could be completely reversed when the children were supplemented with high doses of zinc, thus proving that the symptoms were really caused by a zinc deficiency.

There are some rare forms of zinc deficiency that have genetic causes. The most important form of a zinc-specific genetic disorder is acrodermatitis enteropathica, a rare (incidence 1:500 000) autosomal recessive inheritable disease caused by a mutation in the human zinc importer protein Zip4 (Kury *et al.*, 2002; Wang *et al.*, 2002). This disease is characterized by a zinc-specific malabsorption. The patients suffer from skin lesions, persistent diarrhea, hair loss and multiple infections. They die of infections within a few years if the illness remains untreated but, if diagnosed in time, high-dose zinc supplementation (1 mg/kg/d) can eliminate all symptoms (Neldner and Hambidge, 1975). Another genetic cause for human zinc deficiency is a mutation in the zinc transporter ZnT2, which leads to reduced levels of zinc in breastmilk (Chowanadisai *et al.*, 2006). In this case, the mothers are free of symptoms, whereas the newborns die of zinc deficiency if they do not receive supplements of zinc (Ackland and Michalczyk, 2006). The defect was first described in mice called 'lethal-milk mice' because their pups died after lactation. The defect in mice, however, is caused by a mutation in a different zinc transporter than in humans.

Today, zinc deficiency due to malnutrition as described by Prasad *et al.* (1963) is a common problem in developing countries (further information in Section 5.2.2), whereas it is rare but not absent in industrial nations (Prasad *et al.*, 1993). Here, another type of zinc deficiency is more frequent, the mild, age-related deficiency (see Section 5.4.1). This chapter concentrates on nutritional aspects of zinc intake and its effects on the immune system.

5.2 Nutritional background

Zinc is found throughout the human body, but the highest amounts are found in skeletal muscle, bones, teeth, liver and skin (Kunkle, 1980; Rink and Kirchner, 2000). The total amount of zinc in the human body is about 2–4 g, of which only a small percentage (0.1%) appears in the serum/plasma (10–18 μM , the actual concentration varies with factors like age and gender). In fact, the serum/plasma pool is the smallest in the body, but it is highly important for immunological functions (Mills, 1989; Favier and Favier, 1990). Serum/plasma zinc is mostly bound to albumin (60%), 30% tightly to α_2 -macroglobulin and the rest to other proteins, like transferrin or ceruloplasmin (Rink and Gabriel, 2000). Thus, the amount of zinc which is available for immediate action (also called *free zinc* or *available zinc*) is small, being only a minor fraction of the 0.1% of body zinc found in serum/plasma (Overbeck *et al.*, 2008a). Moreover, the body has no specific storage system for zinc. Therefore zinc homeostasis depends on a tightly regulated steady state of intake and excretion to guarantee a concentration necessary for physiological processes.

In the human body, zinc homeostasis is regulated via five different mechanisms (Hotz *et al.*, 2003; King *et al.*, 2001):

- gastrointestinal absorption and secretion,
- renal excretion,
- release from muscle and skin
- exchange with erythrocytes
- exchange with the liver.

Quantitatively, the most important mechanism is the uptake of orally acquired zinc via the gut and excretion through the faeces (Krebs, 2000). During nutritional zinc deficiency, the excretion of zinc is decreased, whereas the rate of absorption is increased to keep the levels of zinc relatively stable (Lee *et al.*, 1993; Krebs, 2000). Under some circumstances, this balance is disturbed, either by increased elimination or impaired uptake of zinc, resulting in a secondary zinc deficiency. For example, this is the case in patients with diabetes: they have a higher elimination rate of zinc via the urine than healthy people and their levels of plasma zinc are significantly reduced in comparison to healthy subjects (Giannoglu *et al.*, 2010). Another example are patients suffering from chronic inflammatory bowel diseases, who have a disturbed zinc balance due to impaired uptake of zinc via the damaged gut epithelium (Dutta *et al.*, 1998). These patients are at a high risk of developing a secondary form of zinc deficiency and suffer from its complications if they do not receive adequate supplementation.

5.2.1 Assessment of zinc status

In order to correctly evaluate zinc status and/or to diagnose zinc deficiency, serum (or plasma) zinc concentrations are commonly assessed. However, such measurements lack both sensitivity and specificity since, even with insufficient intake, serum zinc levels are maintained at a stable and physiological level for some time.

Only manifest zinc deficiency will alter the serum zinc concentration, and factors like recent meals or stress are strong influencing factors (Hambidge *et al.*, 2002). Another factor which alters the serum zinc concentration is hypoalbuminaemia, for example in patients with chronic renal diseases, since zinc is mostly bound to albumin in the serum and low levels of albumin lead to a reduction of total zinc concentration (Hambidge, 2003). Therefore, when testing for zinc deficiency it is advisable to determine the albumin status of the subject as well. Also, it could be reasonable to check the subject's response to zinc administration, as the serum zinc concentration may not permit a definitive diagnosis (van den Broek, 1986; Colombini, 1999).

Recently, Kanoni *et al.* reported a trial that investigated dietary zinc intake in Europeans aged 60 years and older and its influence on plasma zinc concentrations and cytokine production (Kanoni *et al.*, 2010). They assessed the dietary intake of zinc through a food frequency questionnaire, then calculated a zinc score and found that the zinc scores correlated very well to the plasma zinc concentration. This could be a very sensible additional diagnostic tool, even a good alternative,

but has to be further evaluated before being used as a routine. Another promising approach for exact zinc measurements *in vivo* could be the use of biosensors. They can transform the presence and/or concentration of the desired agent into optical or electrical signals (Thompson *et al.*, 1994). For example, carbonic anhydrase II is used as a transducer and has proved to be effective in exact measurements of free zinc in the range of 0.1–10 ppm. However, up to now a definitive biomarker for the zinc status is missing, since a high number of enzymes are regulated by zinc and the most sensitive to zinc deficiency, e.g. alkaline phosphatase, are altered by a number of other factors (Lowe *et al.*, 2009).

5.2.2 Recommended intake

There are several reasons for a person to become zinc deficient, as described above. Apart from genetic causes and otherwise caused malabsorption, the most prominent and globally most frequent cause of zinc deficiency is malnutrition. According to the Global Health Report (WHO, 2009), zinc deficiency is currently number ten in a ranking of risk factors for high disability adjusted life years and caused 1.5% of deaths in low-income countries (0.7% globally) in 2004. Children are particularly at risk, with about 800 000 child deaths per year and 13% of lower respiratory tract infections (prominently pneumonia and influenza), 10% of malaria episodes and 8% of cases of diarrhea being attributed to zinc deficiency (Bhutta *et al.*, 1999; Sazawal *et al.*, 2001). In industrial countries, the most common form of zinc deficiency is the mild type among elderly people (Chandra, 1997). It causes memory disturbances, a reduced sense of smell and taste, as well as mild night blindness and impaired spermatogenesis (Shankar *et al.*, 1998). It is estimated that over 50% of the elderly people in industrial nations suffer from latent zinc deficiency, and 1–13% of the European and North American population have a severe manifest zinc deficiency (Brown *et al.*, 2001; Haase, *et al.*, 2006).

Zinc is mostly acquired from animal products. Shellfish (especially oysters), red meat, liver, poultry, eggs and dairy products are foods with the highest zinc content (Table 5.1). Since the foods with the highest zinc content are exclusively animal products, it is not surprising, that vegetarians are at a high risk for zinc deficiency (Murphy and Salloway, 1986). However, apart from having a lower zinc content, foods like cereals, legumes, nuts and seeds can also actively impair the uptake of zinc from other foods. This depends on their level of phytates, which chelate zinc and thereby inhibit its absorption in the small intestine (Solomons *et al.*, 1979). Solomons *et al.* showed that if someone ate 120 g of oysters (having the highest zinc content among all foods), their serum zinc content would increase significantly. However, if the same person ate the same amount of oysters along with 120 g of tortillas (made of cornmeal), the serum zinc concentration would not change (Solomons *et al.*, 1979). There are several other factors impairing zinc uptake, among them high amounts of coffee or tea, certain drugs, other metal ions like copper, calcium or iron, metabolic disturbances or chronic illnesses (see Table 5.2) resulting in an increased need for zinc and an elevated risk for zinc deficiency for the person concerned. Therefore, when considering dietary zinc, it is necessary

Table 5.1 Zinc contents of some foods

Plant products	Zinc content (mg/100 g)	Animal products	Zinc content (mg/100 g)
Wholemeal flour	3.0	Oysters	20–150
Rice	1.3	Liver	4–6
Sweetcorn	1.2	Cheese	1–5
Wheat flour	0.9	Calf fillet	4.3
Carrots	0.64	Pork fillet	3.6
Coconut	0.5	Beef fillet	3.6
Sauerkraut	0.32	Pork shoulder	3.5
Potatoes	0.2–0.3	Poultry	2–3
Fruit	0.1–0.3	Roast beef	2.5
Cauliflower	0.23	Fish	1–2
Vegetable oil	0.1–0.2	Pork cutlet	1.3
Salad	0.22	Eggs	0.3–0.5
Red cabbage	0.22	Milk	0.2–0.4
Radish	0.16	Butter	0.15

Source: modified from Ibs and Rink (2004).

Table 5.2 Factors interfering with zinc uptake and risk factors for zinc deficiency

Illnesses	Drugs	Foods	Others
<i>Impaired zinc uptake:</i>		<i>Impaired zinc bioavailability:</i>	
Malabsorption syndromes	ACE inhibitors	Tea, coffee	Age
Intestinal parasitism	Some antibiotics (penicillin)	Deep fried foods	Pregnancy
Chronic enteritis		High fiber foods	Other metal ions:
Chronic pancreatic disease		High phytate foods	iron, copper, calcium
		Raw vegetables	Vegetarian diet
		Corn products	
<i>Heightened loss of zinc:</i>			
Diabetes mellitus	Diuretics	Excess of tea or coffee	
Chronic renal diseases	Chelators like		
Hepatic disturbances	DMPS		
Burns			
Exudative skin illnesses			
Alcoholism			

to take into account foods with high zinc levels, and also to recognize the negative impact of phytate-rich foods and lifestyle factors that influence zinc absorption or secretion.

There are no worldwide standard recommendations for a healthy intake of zinc, and the advised amounts are different among countries. For example, in the USA, the RDA (recommended daily allowance) for people aged 19 years and older is 11 mg for men and 8 mg for women (Maret and Sandstead, 2006). In Germany, the recommendations are 7 mg/d for women and 10 mg/d for men (D-A-CH, 2000), and in the UK 4–7 mg/d for women and 5.5–9.5 mg/d for men (NHS UK, 2012). These differences are a result of the calculation of the intake based on national

eating habits instead of biochemical requirements. This explains why the number of people defined at risk for zinc deficiency is higher in the USA and Germany compared to the UK. In contrast to zinc deficiency, the risk of causing toxic effects with zinc supplementation is relatively low. However, long-term elevated intake of zinc >30 mg/d can lead to secondary copper deficiency and anaemia, as zinc impairs the absorption of copper from the gut (Porter *et al.*, 1977; Prasad *et al.*, 1987; Salzman *et al.*, 2002). Therefore, when supplementing patients with high doses of zinc, their copper status should be assessed at regular intervals to avoid these negative side effects. Interestingly, this effect can be used therapeutically when dealing with Wilson's disease. Here the patients suffer from a disturbed copper metabolism in the liver, meaning they cannot eliminate copper properly and consequently accumulate it in several organs, which causes an intoxication. Zinc can be used in Wilson's disease to inhibit copper uptake and, in contrast to other therapeutics, it does not have severe side effects.

5.3 Molecular mechanisms of zinc action

Zinc has a wide range of functions in cellular metabolism, and about 10% of the proteins encoded by the human genome have zinc-binding motifs (Andreini *et al.*, 2006). Therefore the role of zinc in general cell biology is quite prominent. Zinc is a co-factor for more than 300 enzymes and functions as a catalytic, co-catalytic, structural or regulatory ion, mainly interacting with amino acids such as histidine, glutamic acid, aspartic acid and cysteine (Coleman, 1992; Valle *et al.*, 1993). It is especially important in highly proliferating systems such as the immune system, as zinc finger motifs can be found in many transcription and replication factors. Consequently, during zinc deficiency, cell proliferation is reduced (Rink and Gariel, 2000).

5.3.1 Intracellular zinc

The majority of body zinc is found intracellularly, and 30–45% of the intracellular zinc is located in the nucleus, about 50% in the cytoplasm and other organelles, and the rest in the cell membrane (Rink and Haase, 2007). In order to execute its tasks in cellular signalling, zinc has to be present in its free form.

Therefore, the cell has three main pathways to regulate its homeostasis (Cousins *et al.*, 2006; Rink and Haase, 2007) (see also Fig. 5.1):

- reversible oxidative release from storage proteins;
- uptake from the exterior or release from the cell;
- redistribution between different intracellular compartments.

Increases in the levels of free zinc in the cell are also called 'zinc signals' and generally occur during proliferation and/or stimulation of the cell, so as to allow actions in cell signalling and homeostasis that are dependent on zinc. These zinc signals can generally be divided into three different subtypes that differ in time of appearance and duration (Haase and Rink, 2009):

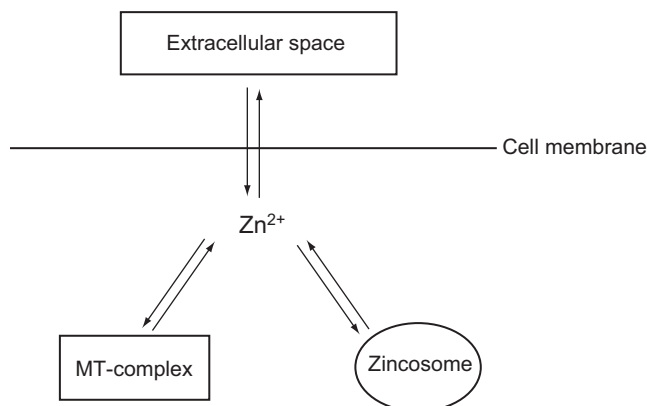


Fig. 5.1 Scheme depicting cellular zinc homeostasis. MT, metallothionein.

1. a fast zinc signal that occurs immediately after stimulation but also fades rather rapidly (Atar *et al.*, 1995; Korichneva *et al.*, 2002; Haase *et al.*, 2008);
2. a so-called zinc wave, which starts a few minutes later, but lasts longer (Hirano *et al.*, 2008);
3. slow zinc signals that start even later, but which also last for hours to days. They usually occur when there are changes in proteins that play a role in zinc homeostasis.

Zinc's main actions in cell-signalling include inhibition of caspases, phosphatases and phosphodiesterases, modulation of protein folding and mediation of protein–protein interactions, all of which will be described more explicitly in Section 5.3.2 (zinc signalling).

Under normal conditions, about 5–20% of the intracellular zinc is bound to MT (metallothionein), a low molecular weight (6 kDa) protein that can bind metal ions, especially zinc, and has protective properties against metal toxicity and oxidative stress (Kägi *et al.*, 1988; Andrews, 2001). Experiments with MT knockout mice have shown that MT is also crucial for the accumulation and storage of zinc in a complex in the liver during inflammation (King *et al.*, 2000). MT knockout mice are more vulnerable to endotoxin-induced shock than the wild type. In this context, MT has been called an acute-phase protein, because its expression increases considerably during inflammatory processes, tissue injuries, neoplastic diseases or sepsis (Coyle *et al.*, 2002). Reversible oxidative release from MT as a storage protein is one of the three ways of keeping a balanced intracellular zinc homeostasis (Fig. 5.1). If it is disturbed, like in the case of MT knockout mice, it affects the cell severely in its functions. Thymocytes of MT knockout mice are impaired in their proliferation and cytokine production upon stimulation with the T cell mitogen Concanavallin A (Sugiura *et al.*, 2004). The expression of MT is regulated by metal response element-binding transcription factor-1 (MTF-1), a sensor of zinc ions (Prasad, 2000b) which is also responsible for the transcription of other

proteins involved in zinc homeostasis, such as the zinc transporter ZnT-1 (Murthy *et al.*, 1993; Kuo *et al.*, 1997).

The other major contributors to the regulation of the intracellular zinc content are a number of zinc transporters, which can generally be divided into two classes in mammals: 14 Zip (Zrt/Irt-like) transporters for zinc import into the cytoplasm and ten ZnT transporters for export to the extracellular space and of zinc sequestration in the cell, by transporting to cell organelles and zincosomes (vesicles for the accumulation of large zinc quantities) (Haase and Beyersmann, 2002; Cousins *et al.*, 2006; Rink and Haase, 2007). The regulation of these transporters occurs via the zinc status, and endocrine and cytokine signalling, as well as direct phosphorylation of the zinc transporter (Aydemir *et al.*, 2006; Cousins *et al.*, 2006; Overbeck *et al.*, 2008b; Taylor *et al.*, 2012). The zinc transporters are important for the uptake and secretion of zinc from the cell as well as for the redistribution of zinc within the cell, especially the redistribution between cell organelles. There have been suggestions that the transferrin receptor and calcium ion channels might be additional non-specific transport matters for zinc but so far, this has not been confirmed (Bentley, 1992; Hogstrand *et al.*, 1996; Wellinghausen *et al.*, 1996). However, some calcium channels are regulated by zinc (Levy *et al.*, 2009).

5.3.2 Zinc signalling

The amount of free zinc in the cell alters during proliferation or stimulation. The signalling function of zinc is mediated by the modulation of enzyme activities, often acting as an inhibitory factor (Haase and Rink, 2009). An overview of intracellular zinc signalling is shown in Fig. 5.2. For example, zinc downregulates protein tyrosine phosphatase activity, leading to a prolonged phosphorylation of proteins in signalling cascades and inhibition of the corresponding transcription factors such as nuclear factor kappa B (NFκB) (von Bülow *et al.*, 2005, 2007; Haase *et al.*, 2008). Phosphodiesterases are also inhibited by zinc. Since these decompose cAMP and cGMP (cyclic adenosine monophosphate and cyclic guanosine monophosphate), their inhibition prolongs the signalling effect of cAMP and cGMP which have direct effects on the activation of monocytes (Wellinghausen *et al.*, 1996). Zinc can mediate the formation of protein complexes, for example by promoting the recruitment of the protein tyrosine kinase Lck to the T cell receptor complex and fastening it there (Huse *et al.*, 1998; Lin *et al.*, 1998; Kim *et al.*, 2003; Romir *et al.*, 2007). This facilitates the interaction between Lck and the receptor which is phosphorylated by it. Moreover, it stabilizes the protein conformation of a few proteins and ensures their functionality by interacting with their tertiary and quaternary structures like in the case of MTF-1 (Berg and Shi, 1996; Auld, 2001). MTF-1 (see also section 1.3.2) is called a 'zinc sensor', because it binds zinc ions with low affinity and binds to its DNA-binding site only in the presence of sufficient amounts of zinc. Recent studies also described a zinc signal during T cell receptor activation (Yu *et al.*, 2011).

Finally, zinc signals can mediate receptor binding, as observed during the investigation of the binding of superantigens to the β-chain of the major histo-

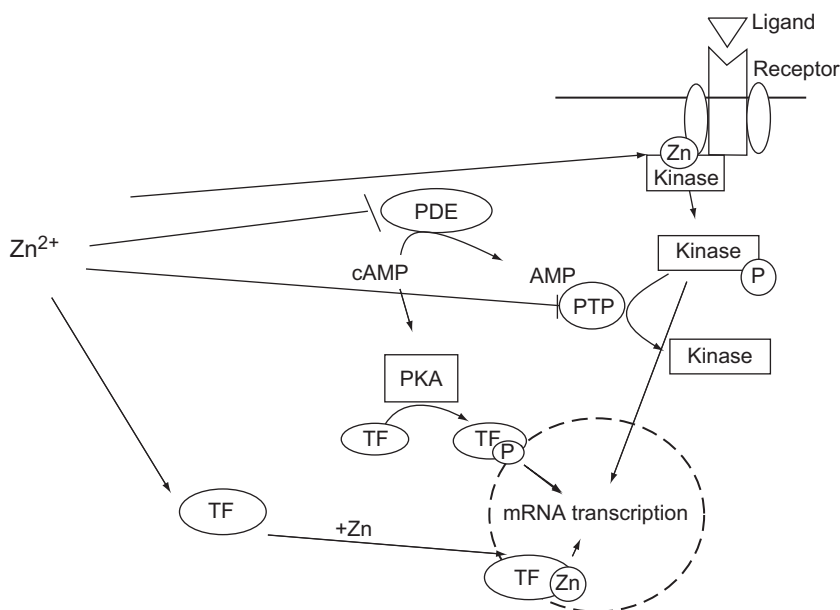


Fig. 5.2 Functions of zinc in cellular signalling. cAMP, cyclic adenosine monophosphate; P, phosphorylated; PDE, phosphodiesterase; PKA, protein kinase A; PTP, protein tyrosine phosphatase; TF, transcription factor; Zn, zinc.

compatibility complex (MHC) class II and in the case of natural killer (NK) cells, which need zinc for the interaction of their p58 killer cell inhibitory receptor (KIR) with MHC I molecules on the target cell (Fraser *et al.*, 1992; Rajagopalan *et al.*, 1995; Bernatchez *et al.*, 1997).

5.3.3 Zinc and apoptosis

Apoptosis is the so-called physiological or programmed cell death, during which the cell undergoes a series of structural changes that lead to core condensation and its fragmentation into apoptotic bodies (Trung-Tran *et al.*, 2001). It is a strictly regulated process and, in contrast to necrosis (supposed to be pathologic cell death), it does not lead to inflammation, since the cell remnants are removed by macrophages and granulocytes. Apoptosis is crucial for the elimination of autoreactive B and T cells during their development as well as in senescence (Rink and Kirchner, 2000). Zinc plays an important role in this process. Depending on its concentration, it can have an inhibiting or even inducing influence on it.

When present in sufficient quantities, zinc's cytoprotective properties prevail, by suppressing apoptosis via inhibition of tumour necrosis factor (TNF)- α -mediated DNA fragmentation, cytolysis and inhibition of caspases 3, 6 and 9, which are important factors in the apoptosis-inducing signalling cascade (Flieger *et al.*, 1989; Trung-Tran *et al.*, 2001; Hershfinkel *et al.*, 2007). When animals are supplemented with zinc, they seem to have an increased tolerance against apoptosis

(Matsuhita *et al.*, 1996; Kuo *et al.*, 1997). Moreover, in a monocyte cell line it has been observed that zinc supplementation increases the Bcl2/Bax ratio (Bcl2 being anti-apoptotic, Bax pro-apoptotic), thus promoting cell resistance against apoptosis (Fukamachi *et al.*, 1998).

However, excessive levels of intracellular zinc can have the opposite effect by causing mitochondrial dysfunction and inducing pro-apoptotic factors like p38 (Trung-Tran *et al.*, 2001; Hershfinkel *et al.*, 2007; Wisemann *et al.*, 2006). In this context, elevated levels of intracellular free zinc seem to play a major part in neuronal death after seizures or ischaemia (Choi *et al.*, 1998; Galasso *et al.*, 2007). Zinc deficiency does also have pro-apoptotic effects. It leads to an elevated number of apoptotic cells in animals *in vivo*, and *in vitro* studies have shown a pro-apoptotic effect of the treatment of cell cultures with the zinc chelator TPEN (N,N,N',N'-tetrakis-2-pyridylmethyl-ethylendiamine) (Zalewski and Frobes, 1991; Zalewski *et al.*, 1993; Treves *et al.*, 1994). Other experiments revealed a synergistic effect of zinc depletion on pro-apoptotic substances (Zalewski *et al.*, 1993; Meerarani *et al.*, 2000), meaning that during zinc deficiency, cells are more susceptible to apoptosis caused by toxins. Dysregulated apoptosis due to zinc deficiency might therefore be of importance in abnormal embryonic development and thymic function (Fraker, 2005), as well as in diseases such as diabetes mellitus or Alzheimer's disease, as increased apoptosis rates come with an elevated risk for these diseases and patients may suffer from undetected zinc deficiency (Trung-Tran *et al.*, 2001).

Among zinc's cell-protective functions are its antioxidative capacities. It can protect lipids, cell-membrane proteins and thiol-dependent macromolecules (among them several enzymes) directly from damage inflicted by free radicals. Zinc inhibits NADPH-oxidases and other enzymes responsible for production of superoxide radicals and is a component of the antioxidant enzyme Cu/Zn superoxide dismutase (Chausmer, 1998; Prasad, 2008). Thus, zinc deficiency may lead to increased oxidative mitochondrial decay, which is an acceleratory factor for ageing (Beckmann and Ames, 1998).

Taken together, it seems that the effects of zinc on apoptosis vary with its concentration: not only the lack of zinc, but also too high levels of zinc can cause cell death. This pattern recurs in all aspects of zinc's immunological actions and makes it clear that for optimal functionality, the levels of zinc have to be kept within a narrow range.

5.4 Effects of zinc on immune function

The immune system is delicately regulated by zinc. Zinc influences the non-specific innate immune system as well as the specific adaptive immune system. Since both parts work together in an intact immune response, overall immune capacity is reduced during zinc deficiency. The effects of zinc deficiency on the immune system are summarized in Table 5.3. However, an excess of zinc leads also to disturbed immune functions, especially in T cells. The effects of zinc excess on the immune system are also summarized in Table 5.3.

Table 5.3 Effects of zinc deficiency and zinc excess on immune cells

	Zinc deficiency	Zinc excess
<i>Innate immunity</i>		
NK cells	Numbers ↑ Cytotoxicity ↓ Unspecific killing ↑ (inhibition via KIR receptors ↓)	Killing ↓ (probably elevated risk of tumour growth)
Monocytes Phagocytosis ↓ Adhesion ↓	Activation ↓	>100 µM direct activation
Granulocytes	Cell numbers ↓ Phagocytosis ↓ Oxidative burst ↓ Chemotaxis ↓	>500 µM direct chemotactic activity
<i>Specific immunity</i>		
B cells	Apoptosis ↑ (precursor cells ↓) Antibody production ↓	Apoptosis ↑
T cells	Cell numbers ↓ (Thymulin ↓) Th1/Th2 ratio ↓ (IL-2 and IFN-γ production ↓) Autoreactivity ↑	>30 µM reduced functions >100 µM suppressed functions

5.4.1 Effects of zinc deficiency in elderly people

Ageing is generally associated with a deterioration of immune functions, leading to an increased number of infections, neoplasia and autoimmune diseases. As these immunological symptoms are very similar to those observed during zinc deficiency, plasma zinc levels of elderly people have been assessed to see if there was a connection: many elderly subjects showed mildly reduced zinc levels (Cakman *et al.*, 1997; Briefel *et al.*, 2000; Kahmann *et al.*, 2006). Mild zinc deficiency among the elderly is no rarity (Prasad *et al.*, 1993; Briefel *et al.*, 2000; Haase *et al.*, 2006). A study from 1971 examined plasma zinc levels of male and female subjects aged 20–84 years and found a significant inverse relationship (Lindeman *et al.*, 1971). Possible reasons for this decline could be an altered diet, changes in intestinal absorption during ageing, or possible side effects of drugs such as diuretics that are responsible for a negative zinc balance.

The reduced zinc levels have multiple effects on the immune system. They lead to a dysregulated basal release of pro-inflammatory cytokines, causing a status of low-grade inflammation and an impaired immune response (Kahmann *et al.*, 2008: see Section 5.5). Furthermore, zinc deficiency affects the different subsets of immune cells in many ways.

While short-term effects include most notably the reduction of thymulin activity (thymulin is a thymic hormone necessary for T cell activation) and increased maturation of macrophages (Dubben *et al.*, 2010), long-term effects include changes in all leukocyte subpopulations (Fraker and King, 2004: see

Sections 5.4.2 and 5.4.3). In mice, zinc deficiency during pregnancy was even observed to impair immune functions in the second and third generation showing that zinc changes the immune response at an epigenetic level (Beach *et al.*, 1982).

5.4.2 Innate immunity

Innate immunity is responsible for the first, rapid but non-specific immune response. The cells of this part of the immune system, namely macrophages and granulocytes, recognize PAMPs (pathogen-associated molecular patterns) which are characteristic for the respective pathogen, via pattern-recognition receptors such as the TLRs (Toll-like receptors). The development of innate immune cells is dependent on zinc, as they all derive from bone marrow stem cells which need zinc for growth and maturation (Ibs and Rink, 2003). Apart from the immune cells, there are additional means of protection against pathogen invasion, such as the physical barriers presented by the epithelial cells of the skin, and the respiratory and gastrointestinal tracts. These also need zinc to remain intact and functional, like every highly proliferating system (Shankar and Prasad, 1998). However, it is not only human cells that require zinc; pathogens are also severely impaired in their growth if deprived of it. Thus, during infection, neutrophil granulocytes locally release calprotectin, a substance that chelates zinc and so inhibits the replication of bacteria (Rink and Gabriel, 2000; Rink and Kirchner, 2000). Furthermore, some antimicrobial peptides are activated by zinc (Poiraud *et al.*, 2012). During zinc deficiency, functions of all cells representing innate immunity are affected.

Reduced zinc levels during the acute-phase response induced macrophage maturation (Dubben *et al.*, 2010), whereas in long-term zinc deficiency macrophages and neutrophils show reduced phagocytosis and generation of the oxidative burst *in vivo* (Allen *et al.*, 1983; Keen *et al.*, 1990). The number of NK cells is elevated, but their specific killing rates are reduced. The body tries to compensate for the reduced killing activity by increasing the number of NK cells (Rink and Seyfarth, 1997; Rink *et al.*, 1998).

Under conditions of zinc deficiency, the cells of the innate immune system also show altered cytokine expression. In zinc-deficient elderly persons, monocytes showed an increased secretion of pro-inflammatory cytokines when stimulated with lipopolysaccharides (LPS) or phorbol ester and the number of pre-activated T cells was elevated (Fagiolo *et al.*, 1993; Rink *et al.*, 1998). Thus, zinc might also be involved in the negative regulation of cytokine production, and deficiency could lead to an over-production (Haase and Rink, 2009). Zinc substitution, on the other hand, led to a reduction of the spontaneous cytokine release and improved the response to PAMPs, and it also restored the disturbed interferon (IFN)- α production of peripheral blood mononuclear cells (PBMC) of zinc-deficient elderly (Cakman *et al.*, 1997; Kahmann *et al.*, 2008). For interleukin (IL)-1 at least, it was shown that the pro-inflammatory effect of zinc deficiency is mediated on an epigenetic level (Wessels *et al.*, 2013).

Thus, under zinc-deficient conditions, monocytes have an increased basal activity and non-specifically produce pro-inflammatory cytokines, causing a

constant low-level inflammation, which might cause damage to the DNA via induction of reactive oxygen species over time and increase the risk for tumour growth. On the other hand, the pro-inflammatory cytokine response upon challenge is reduced in comparison with a zinc-sufficient control group so that, in case of infection, the immune response is less effective (Kahmann *et al.*, 2008). This might be one of the reasons why elderly people are more prone to chronic inflammatory and neoplastic diseases, whereas their response to infections is impaired. There is one study from Hujanen *et al.* (1995) that described zinc acting as a chemotactic agent for neutrophils, but so far this could not be confirmed by others. Recent studies indicated that zinc is important for NET (neutrophil extra-cellular traps) production by neutrophils (Hasan *et al.*, 2013).

In addition to affecting monocyte function, zinc deficiency also affects their numbers. In mice with mild and severe zinc deficiency, the numbers of monocytic cells in the myeloid compartment of the bone marrow were increased by approximately 80% compared to non-zinc-deficient mice (King and Fraker, 2002). Furthermore, the subset of proliferating monocytes was raised by about 40%, and *in vitro* it has been shown that zinc deficiency increased the differentiation of a promyeloid cell line to macrophages (King and Fraker, 2000; Dubben *et al.*, 2010). This means that under zinc-deficient conditions the total number of monocytes (and granulocytes) as well as their rate of proliferation is increased, but their function is reduced.

5.4.3 Adaptive immunity

T cells

T cells are the group of immune cells which is most dependent on zinc. It is not only necessary for their maturation, but also for keeping them functional in their mature state. T cell precursors are derived from the bone marrow but mature in the human thymus, a process that is dependent on the hormone thymulin which is secreted by thymic epithelial cells and induces the differentiation of immature T cells (Saha *et al.*, 1995).

The biologic activity of thymulin is controlled by zinc, and even small changes in plasma zinc levels can impair it severely, so that without zinc the proliferation rates in T cells are reduced (Prasad *et al.*, 1988, 1998b, 2008). Apart from directing their maturation, thymulin also affects peripheral T cells. First, it induces and enhances the expression of the high-affinity receptor for IL-2 in mature T cells (Tanaka *et al.*, 1989), which is an important step in their activation. Second, it modulates cytokine production by PBMCs and thereby induces proliferation in CD8+ cells in cooperation with IL-2 (Coto *et al.*, 1992; Safie-Garabedian *et al.*, 1993). Thus, it is not surprising that during severe zinc deficiency the proliferation of T cells upon mitogenic stimulation is decreased (Dowd *et al.*, 1986; Crea *et al.*, 1990).

Generally, of all immune cells, T cells seem to be the most susceptible to zinc deficiency. The numbers of peripheral and thymic T cells are decreased, as well as

the function of cytotoxic and helper T cells (Fraker and King, 2004). One possible explanation for the decreased T cell activity could be the impairment of the effect of IL-2 during zinc deficiency. Recently, Kaltenberg *et al.*, investigated the impact of zinc on IL-2 signalling and found that a zinc signal is triggered upon binding of IL-2 to its receptor. This zinc signal is necessary for the IL-2 induced proliferation in T cells, as it impairs the dephosphorylation of the signalling molecule ERK (Kaltenberg *et al.*, 2010). So when cellular zinc levels are low, the proliferative effect of IL-2 might also be impaired. Furthermore, IL-1-induced T cell activation depends on the concentration of intracellular free zinc (Daaboul *et al.*, 2012).

Low zinc levels can also cause a disturbance in the Th1/Th2 balance in favour of Th2 cells. On the one hand, Th1 cell products such as IFN- γ and IL-2 are decreased; on the other hand, Th2 cell products such as IL-4 and IL-10 are not affected (Cakman *et al.*, 1996; Prasad, 2000b). Thus, the downregulation of Th2 cells by IFN- γ is impaired, whereas the suppression of Th1 cells by IL-10 is not affected and the Th1/Th2 ratio decreases significantly (Beck *et al.*, 1997; Cakman *et al.*, 1996; Prasad, 1998; Prasad, 2000b). Keeping a balance in the Th1/Th2 system is important for an appropriate immune response during infection, because the cells have different functions. Th1 cells are involved in the elimination of intracellular pathogens, via stimulation of macrophages and granulocytes, whereas Th2 cells act more against extracellular pathogens by prompting B cells to enhance their antibody production. So, if this system is disturbed, and Th1 cells are downregulated during zinc deficiency, difficulties in the elimination of intracellular pathogens are the consequence. However, zinc supplementation can reverse these effects, as it leads to significantly increased rates of IFN- γ release from human PBMCs and, because IFN- γ is one of the main inducers of Th1 cells, it contributes to restoring the Th1/Th2 balance (Kahmann *et al.*, 2008). Recently, it could be shown that the increased IFN- γ production is mediated by a zinc-dependent activation of p38 leading to phosphorylation of the transcription factor cAMP response element-binding protein (CREB), which enhanced IFN- γ and IL-2 transcription (Hönscheid *et al.*, 2012). On the other hand, excessive zinc levels can also have a suppressing effect on T cells, in this case by inhibiting the IL-1-receptor-associated kinase and thus avoiding IL-1-dependent T cell activation (Wellinghausen *et al.*, 1997).

B cells

Zinc deficiency affects B cells less than T cells but influences their development nonetheless (Fraker *et al.*, 1993; Fraker *et al.*, 1995; Fraker and Telford, 1997). During zinc deficiency the absolute numbers of B cells are reduced, those of immature and precursor B cells more pronouncedly than those of mature B cells (Fraker *et al.*, 1995). This is due to the general impact of zinc on apoptosis, as zinc deficiency increases the rate of apoptosis during B cell development. In this context, it could be shown that glucocorticoids, secreted in response to zinc deficiency, provoke an elevated rate of apoptosis in immature B and T cells in the bone marrow and thymus, respectively (Fraker *et al.*, 2000; King *et al.*, 2002).

Furthermore, low zinc levels lead to a reduced antibody production by B lymphocytes, especially in response to T cell-dependent antigens (Fraker *et al.*,

1978; Fraker, 1983; Depasquale Jardieu *et al.*, 1984; Moulder *et al.*, 1989). Moreover, the immunological memory is affected, since in experiments with zinc deficient mice it was shown that their antibody responses were reduced, for T cell dependent as well as for T cell independent antigens (Fraker, 1978; Depasquale-Jardieu, 1984; Fraker *et al.*, 1986, 1987).

5.5 The impact of zinc in inflammatory processes

During inflammation, the zinc level in the human body undergoes considerable changes. In response to endotoxins such as LPS, the plasma zinc concentration decreases as zinc moves into organs, in this case mostly into the liver (Cousins *et al.*, 1988). This happens mainly via the zinc importer Zip14, whose expression increases in response to pro-inflammatory cytokines such as IL-6. The amount of MT is also increased, so that the overall concentration of zinc in the liver is generally higher during inflammation, but free zinc increases much less dramatically (Schroeder *et al.*, 1990; Coyle *et al.*, 1995). This could be a mechanism of protection of the liver, as it is at a high risk of being damaged during inflammatory processes. In fact, during sepsis, where cytokine production is abnormally high and out of control, most people die of multi-organ failure, including hepatic failure. LPS, which activates TLR4 (Toll-like receptor 4, a pattern-recognition receptor of the IL-1 receptor family on cell surfaces) and leads to initiation of the immune response is often responsible for triggering the so-called 'cytokine storm' that causes the septic reaction (Rink and Haase, 2007). Zinc can enhance the cytokine response when given contemporaneously with LPS by altering its structure but, when administered before stimulation with LPS, it can help to circumvent hepatotoxicity and endotoxic shock (Snyder and Walker, 1976; Shankar and Prasad, 1998; Rink and Gabriel, 2000; Rink and Kirchner, 2000:). This could be of interest for example in diseases like bacterial meningitis, where the endotoxic shock is a much feared complication, and zinc could be a new option for protection.

The latter protective effects may be due to the fact that zinc is responsible for inhibition of phosphodiesterases (PDEs) that degrade cGMP, which in turn inhibits the release of TNF- α and IL-1 β (von Bülow *et al.*, 2005). So increased zinc levels lead to elevated levels of cGMP and consequently to reduced levels of the pro-inflammatory cytokines TNF- α and IL-1 β which are partly responsible for the damage inflicted in the liver and other organs during inflammation.

When supplemented with high doses of zinc, IL-6, IL-1, sIL-2 and IFN- γ were released by human PBMCs *in vitro* (Salas *et al.*, 1987; Driessen *et al.*, 1994), whereas the stimulation of isolated T and B cells showed no effect (Crea *et al.*, 1990; Hadden, 1995; Wellinghausen *et al.*, 1997). These results suggest that the activation of lymphocytes requires monocytes and their cytokines (IL-1, IL-6), along with cell-cell contact (Driessen *et al.*, 1994; Wellinghausen *et al.*, 1997). Again, the effect of zinc on cytokine expression seems to be dependent on the dosage. While moderate zinc supplementation leads to an enhanced cytokine

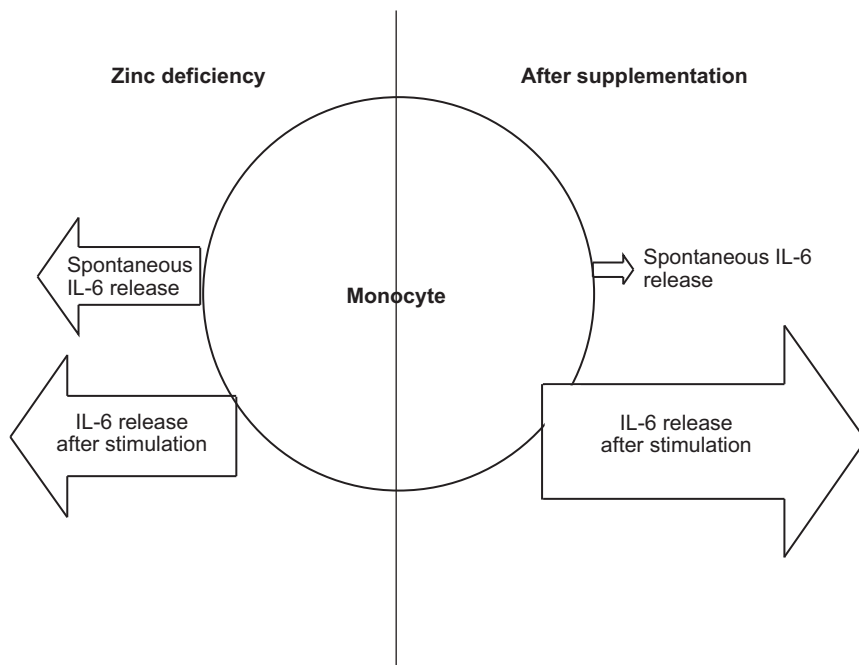


Fig. 5.3 Effects of zinc supplementation on IL-6 release from monocytes.

production by PBMCs, higher amounts seem to suppress it, probably via inhibition of PDEs and subsequent activation of PKA (protein kinase A) (von Bülow *et al.*, 2005, 2007). As mentioned above, elderly people often have reduced zinc levels and disturbed immune functions, including a dysregulated IL-6 release (see Section 5.4.1). However, when these persons were supplemented with zinc, and their plasma and intracellular zinc levels had risen, this dysequilibrium could be corrected. Spontaneous release of IL-6 ceased nearly completely, whereas the specific production after stimulation with LPS increased significantly (Fig. 5.3) (Kahmann *et al.*, 2008). Kahmann *et al.*, come to the conclusion that zinc supplementation of zinc-deficient elderly subjects reduces spontaneous inflammatory processes, leading to a lower number of unspecifically activated cells, and ameliorates the immune response against pathogens. The molecular processes involved are still not completely clear, but it can be assumed that the higher zinc levels impair the release of some pro-inflammatory cytokines via inhibition of PDEs (von Bülow *et al.*, 2005) and that zinc changed the promoter accessibility of pro-inflammatory cytokines (Wessels *et al.*, 2013).

5.6 Zinc in human health and clinical nutrition

Zinc deficiency is a major problem in developing countries, but also of significance in industrialized areas, most prominently among the elderly population, as

discussed before. Fortunately, all negative effects can be reversed by zinc supplementation. When a group of elderly zinc-deficient people were supplemented with zinc for 12 months until their plasma zinc levels were normal, their numbers of T and NK cells as well as IL-2 and sIL-2R production increased significantly. Moreover, in comparison to a group treated with a placebo, their lymphocyte response to stimulation with PHA (phytohaemagglutinin) and NK cell activity were improved considerably (Chandra, 1992). Another study found that after supplementation with 25 mg/d of zinc for three months not only did the cell-mediated immune response improve, but the amount of CD4+ and cytotoxic T cells also increased significantly (Fortes *et al.*, 1998). Thus, zinc supplementation seems to have beneficial effects.

The danger of causing toxic effects by zinc supplementation is relatively small. Ingestion of potentially toxic amounts of zinc leads mostly to vomiting and thus an acute intoxication is prevented. Long-term intake of high levels of zinc could cause copper deficiency (see Section 5.2.2), but that can be avoided by regular monitoring of copper levels or additional copper supplementation (Prasad, 1979). In studies dealing with long-term zinc-supplemented patients, no side effects other than copper deficiency were observed.

In a trial called AREDS (age-related eye disease study), patients were given doses of 80 mg zinc oxide per day (eight times the RDA) over an average period of 6.3 years to see if it had any effect on progression and outcome of their age-related lens opacities and visual acuity loss. The combination of high-dose antioxidants and zinc brought a statistically significant reduction of the risk of developing advanced age-related macular degeneration, and there were no side effects of the long-term supplementation. Copper deficiency was circumvented by an additional 2 mg/d copper as cupric oxide (AREDS, 2001). In an *in vitro* study, concentrations corresponding to three to four times the physiological plasma zinc level neither impaired T cell proliferation nor had any immunosuppressive effects, but suppressed alloreactivity in the mixed lymphocyte culture (MLC) (Campo *et al.*, 2001). The inhibition of the MLC was also possible if donors tested for allogenic response were supplemented with 80 mg zinc per day for one week (Faber *et al.*, 2004). This latter finding indicates a possible new therapeutic use for zinc for immunosuppression in T cell-mediated reactions such as rheumatoid arthritis or in transplantation medicine. In a rat transplantation model the graft survival was significantly increased due to zinc supplementation (Kown *et al.*, 2002). This is in agreement with the observations of others showing an immunosuppression at zinc doses of 100 mg/d, namely suppressed T cell functions, increased apoptosis rates in B cells and suppressed killing activity of NK cells (Brodersen *et al.*, 1995; Provinciali *et al.*, 1998; Turk *et al.*, 1998).

To summarize the current literature on zinc supplementation, it seems to be safe to treat patients who are under suspicion of being zinc deficient with a dosage of 10 mg/d. At this intake, no side effects have been reported or are to be expected. Another study dealing with the effects of zinc on cytokine production showed that in healthy elderly people who were given 10 mg zinc as aspartate per day, the spontaneous release of pro-inflammatory cytokines was significantly reduced

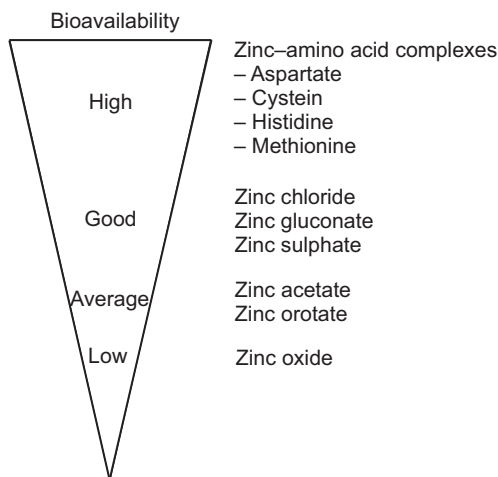


Fig. 5.4 Bioavailability of zinc salts. Modified from Brieger and Rink (2010).

(Kahmann *et al.*, 2008). So even in healthy elderly subjects or people at high risk for deficiency, this dose can be given prophylactically (Brieger and Rink, 2010).

5.6.1 The effects of zinc on various diseases

Many clinical trials have examined the effects of zinc in a large variety of diseases (Table 5.4), but the results are often inconsistent and contradictory, as there are several problems to be considered. First, zinc salts have very different bioavailabilities (Fig. 5.4), and there was no standard supplement for all the studies. Second, the zinc status of the patients was often not properly assessed and documented, so that the trials differed considerably in their settings and also in their results (Aggett, 2001; Mittmann, 2001; Haase *et al.*, 2006). Third, problems with zinc absorption can occur, due to hypo- or achlorhydria as often observed in elderly patients, as zinc uptake is generally enhanced by gastric acidity (Henderson *et al.*, 1995). Additionally, zinc can interfere with the uptake of other metal ions, as documented for iron and copper (see also Section 5.2.2) (Olivares *et al.*, 2007).

Viral infections

Probably the most common viral disease in humans is the common cold, and therefore this is a popular target for research. Many studies have investigated the therapeutic effect of zinc, but the results vary considerably. Despite this, a review by Hulisz (2004) concluded that, when administered within 24 hours after onset of symptoms, it can be effective in reducing duration of symptoms (Hulisz, 2004). Also, in a study where zinc doses of 15 mg/d were administered preventatively over seven months, zinc reduced the incidence of the common colds in children (Kurugöl *et al.*, 2006).

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Table 5.4 Effect of zinc on various diseases (modified from Overbeck *et al.*, 2008)

Disease	Studies	Effects	References
Common cold	> 12 studies, 4.5–23.7 mg/d	Duration of symptoms ↓ if administered within 24 h after beginning	Hulisz, 2004
HIV	7 studies, 10–50 mg/d elemental zinc over 6 d–6 mo	Stabilization of body weight Opportunistic infections ↓ CD4 ⁺ cells ↑ No effect on virus load No effect on CD4/CD8 ratio No improvement in immune response to tuberculosis No improvement in response to pneumococcal vaccine No effect on birth outcomes in pregnant women Risk of wasting ↑	Mocchegiani <i>et al.</i> , 1995 Zazzo <i>et al.</i> , 1989 Bobat <i>et al.</i> , 2005 Green <i>et al.</i> , 2005 Fawzi <i>et al.</i> , 2005 Villamor <i>et al.</i> , 2006 Deloria-Knoll <i>et al.</i> , 2006
Hepatitis C	2 studies, 2 × 17 and 5 × 50 mg/d elemental zinc over 24 wk and 6 mo, respectively	Response to IFN-α therapy ↑ Gastrointestinal disturbances ↓ Body-weight loss ↓ Mild anaemia ↓	Takagi <i>et al.</i> , 2001 Ko <i>et al.</i> , 2005
Diarrhea	Multiple studies with different settings	Duration ↓ Occurrence ↓ Severity ↓	Hoque and Binder, 2006
Lepromatous leprosy	4 studies, 200–220 mg/d over 13 wk–18 mo	Required drug doses ↓ Incidence, severity, duration and size of erythema nodosum granuloma ↓ Number of lymphocytes ↑ Neovascularization ↑ Endothelial cell proliferation ↑	Mathur <i>et al.</i> , 1983 Mathur <i>et al.</i> , 1984 and El-Shafei <i>et al.</i> , 1988 Mahajan <i>et al.</i> , 1994

(Continued)

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Table 5.4 Continued

Disease	Studies	Effects	References
Tuberculosis	1 study, 15 mg/d daily over 6 mo	Plasma retinol concentrations ↑ Earlier sputum conversion Earlier resolution of X-ray lesion areas	Karyadi <i>et al.</i> , 2002
Acute pneumonia	2 studies, 10–20 mg/d over 5 d–6 mo	Episodes of infection ↓ Recovery from illness and fever in boys ↓	Sazawal <i>et al.</i> , 1998 Mahalanabis <i>et al.</i> , 2004
Malaria	5 studies, 10–40 mg/d over 4 d–15 mo	<i>P. falciparum</i> -induced febrile episodes ↓ Trend to reduction of malaria episodes	Shankar <i>et al.</i> , 2000 Bates <i>et al.</i> , 1993 Müller <i>et al.</i> , 2001
	Zinc against plasmodium studygroup, 2002	Prevalence of diarrhea ↓ No effect on parasitaemia and fever	Richard <i>et al.</i> , 2006
Leishamaniosis	1 study, 0.83, 1.67, 3.33 mg/kg/d over 45 d	Cure rates ↑ Erythema ↓ Size of induration ↓	Sharquie <i>et al.</i> , 2001
Rheumatoid arthritis	5 studies, 45–660 mg/d over 15 d–24 mo	Joint swelling ↓ Morning stiffness ↓ Walking time ↑ No antirheumatic activity or other improvements Capacity of releasing reactive oxygen species by monocytes <i>in vitro</i> ↓ Phagocytic activity of PBMC ↑	Simkin <i>et al.</i> , 1976 Mattingly and Mowat, 1982 Rasker and Kardaun, 1982 Herold <i>et al.</i> , 1993 Peretz <i>et al.</i> , 1994

Source: modified from Overbeck *et al.* (2008a).

Zinc has also been tested as an additional therapeutic in HIV therapy (Coovadia *et al.*, 2002). The first study found increased phagocytosis by polymorphonuclear neutrophils, an augmentation of HLA-DR positive macrophages and improved lymphocyte proliferation in response to stimulation with PHA and Concanavalin A (Zazzo *et al.*, 1989). Others found a reduced frequency of candida and *Pneumocystis jirovecii* infections, as well as an increase in Th cell numbers (Mocchegiani *et al.*, 1995). On the other hand, another study was not able to find any changes in the CD4/CD8 ratio after zinc supplementation (Zazzo *et al.*, 1989). Furthermore, Fawzi *et al.* investigated the effects of 25 mg zinc per day over six months in HIV-positive pregnant women and there was no effect on pregnancy outcome, but sometimes a significantly lower red blood-cell count, packed cell volume and lower increases in haemoglobin after delivery (Fawzi *et al.*, 2005). Two studies even found an increased risk for progression to AIDS and reduced survival rates in HIV-positive men who acquired high amounts of zinc via food and/or supplements in comparison to men with lower intakes (Tang *et al.*, 1993, 1996).

There are several reports of zinc having positive effects on hepatitis C. Less gastrointestinal disturbances, body weight loss and mild anaemia were found in patients with a chronic form of this disease. Co-therapy with 75 mg zinc two times a day was several times more effective than the IFN- α -therapy alone (Takagi *et al.*, 2001; Ko *et al.*, 2005). In this context, a study from Berg *et al.* confirmed that the antiviral effects of human IFN- α were potentiated tenfold by addition of zinc *in vitro*, but the effect has yet to be reproduced *in vivo* (Berg *et al.*, 2001).

Bacterial infections

Zinc has been assessed as a therapeutic option in many studies concerning diarrhea, and it has been proved that it can lead to a reduction in severity and incidence of the disease (Fischer *et al.*, 2004; Hoque *et al.*, 2006). In developing countries, prophylactic zinc administration reduces both prevalence and incidence of diarrhea in children (Bhutta *et al.*, 1999, 2000). When administered in acute or persistent cases, the duration of diarrhea was considerably shorter and the rates of treatment failure were significantly reduced by 42%. Apart from immunological effects, zinc improves water and electrolyte absorption in the intestine and accelerates the regeneration of the gut epithelium, effects that might be important in the context of diarrhea (Bettger *et al.*, 1981; Ghishan, 1984; Patrick *et al.*, 1987; Hoque *et al.*, 2006).

Several illnesses are associated with low zinc levels, for example leprosy and tuberculosis (George *et al.*, 1991; Ciftci *et al.*, 2003). In these cases, beneficial effects of zinc supplementation were reported (Mathur *et al.*, 1983, 1984; El Shafei *et al.*, 1988; Mahajan *et al.*, 1994). Both diseases are caused by mycobacteria (*Mycobacterium leprae* and *Mycobacterium tuberculosis*), and the positive effect of zinc might be due to its ability to restore the Th1/Th2 balance, especially the IFN- γ induced immune functions, as Th1 cells are required to eliminate intracellular bacteria (such as mycobacteria). As mentioned earlier, zinc deficiency causes a Th2-shift and a consequent decrease of IFN- γ (Cakman *et al.*, 1996; Beck *et al.*, 1997; Prasad, 2000).

In addition, zinc seems to be efficient in preventing pneumonia. A pooled analysis of four trials investigating the effect of continuous zinc supplementation confirmed that the incidence of pneumonia in children living in developing countries was reduced by 41% (Bhutta *et al.*, 1999). Another study conducted among residents of a nursing home found low zinc levels to be associated with higher rates of pneumonia, so that zinc could also be a possible approach for prevention in the elderly (Meydani *et al.*, 2007).

Parasitic infections

Patients with an acute case of malaria have decreased plasma zinc levels in response to the acute-phase reaction (Duggan *et al.*, 2005). However, the results from different studies investigating possible effects of zinc on malaria are contradictory. Two studies reported positive effects of zinc, meaning fewer febrile episodes in *Plasmodium falciparum* infections and fewer malaria episodes in general (Bates *et al.*, 1993; Shankar *et al.*, 2000). However, the latter effect was not significant. Two other studies did not see any effect of zinc on malaria (Müller *et al.*, 2001; Richard *et al.*, 2006).

Another parasitic infection on which the therapeutic effect of zinc was tested is acute cutaneous leishmaniasis. It is caused by different subtypes of leishmania, which are mostly transmitted by sandflies, and manifests itself in chronic skin ulcers. It seems that patients suffering from leishmaniasis have decreased plasma zinc levels and, when supplemented with zinc, increased cure rates along with an alleviation of the size of the induration and of the erythema were observed (Van Weyenbergh *et al.*, 2004; Sharquie *et al.*, 2001). Another study found zinc to have a direct inhibitory effect on different leishmanial enzymes (Al-Mullah Hummadi *et al.*, 2005). However, the physiological importance of these observations remains to be confirmed, as the effects were only seen at supraphysiological zinc levels of 150 µM and more.

Autoimmune diseases

Many autoimmune diseases like diabetes mellitus type 1 or rheumatoid arthritis (RA) are mediated by T cells that recognize surface antigens on the body's own cells and attack them. In Section 5.4.3 it was described that T cell functions, along with NK cell functions and the development of B cells, are inhibited by high levels of zinc, and these findings indicate that there is a possible therapeutic use of zinc against autoimmune diseases (Rink and Kirchner, 2000; Ibs and Rink, 2003). In fact, there have been many trials concerning the effect of zinc on diseases like RA but, so far, there has been little success, although RA patients have reduced serum zinc levels and the zinc level was shown to correlate negatively with amounts of TNF- α , IL1- β and acute-phase proteins (Mattingly *et al.*, 1982; Rasker *et al.*, 1982; Bao *et al.*, 2003). However, a recent Japanese study found that in mice zinc suppressed the development of Th17 cells, a subset of CD4⁺ T cells that produce pro-inflammatory cytokines such as IL-17A and are the main protagonists of developing T cell-mediated autoimmune diseases. The lack of IL-17A leads to protection against autoimmune diseases like CIA (collagen-induced arthritis) or

EAE (experimental autoimmune encephalomyelitis) (Cua *et al.*, 2003; Harrington *et al.*, 2005; Park *et al.*, 2005; Veldhoen *et al.*, 2006; Bettelli *et al.*, 2007). The results might be interesting for further clinical research, as the development of CIA was successfully inhibited with supraphysiologically high zinc dosages (sera from zinc-supplemented mice had an average zinc concentration of 29 μM , which is twice normal), so that it remains to be seen if the results can be exploited for therapeutic use (Kitabayashi *et al.*, 2010). Furthermore, zinc inhibited only the development of Th17 cells from CD4⁺ T cells but had no effects on already present pathogenic Th17 cells (Kitabayashi *et al.*, 2010), meaning that its possible role at present seems to be more prophylactic than curative. Lastly, if the doses in this mouse model are adapted to humans, the amounts are in a region of those showing clear immunosuppressive effects and therefore are not usable for prophylactic strategies. However, a recent study showed that physiological zinc doses of 0.3 or 1.5 mg/kg body weight also reduced the severity of EAE, which acts as a mouse model for multiple sclerosis (Stoye *et al.*, 2012).

Vaccination

Zinc deficiency impairs antibody production (Depasquale-Jardieu *et al.*, 1984; Fraker *et al.*, 1986; Fraker *et al.*, 1987; Kreft *et al.*, 2000), but currently, only one study could report positive effects of zinc supplementation on the outcome of vaccinations (Duchateau *et al.*, 1984). This may be due to the fact that, although zinc is able to reconstitute normal Th function, high doses inhibit T cells directly (Wellingtonhausen *et al.*, 1997). In one study, the patients had to take zinc for a month before tetanus vaccination, but stopped the supplementation during vaccination in order to avoid the direct inhibitory effect (Duchateau *et al.*, 1981). A study concerning vaccination against cholera also found positive effects (Albert *et al.*, 2003), but was contradicted by another one that found negative effects (Qadri *et al.*, 2004).

5.7 Conclusion and future trends

It is evident, that zinc plays an important role in human health in general. From the multitude of symptoms and disturbances occurring during zinc deficiency, it is clear how widespread the functions of zinc are. It has been shown that zinc is necessary for keeping the immune system functional, from the development of precursor cells and the regulation of the activity of mature ones to directing inflammatory processes. Hence it is not surprising, that there have been many trials using zinc to manipulate the immune response. Zinc supplementation has been proven successful in preventing or reversing effects of zinc deficiency, a problem that is quite common in the elderly and is getting more relevant, as the percentage of seniors is rising in western societies. Moreover, trials investigating the effect of zinc in preventing illnesses like pneumonia or diarrhea in countries with high malnutrition rates showed effective results, especially in children. So zinc supplementation for people with, or at high risk for, zinc deficiency seems sensible. The

finding that high dosages of zinc inhibit the MLC seems to be very promising for transplantation medicine, as there are no side effects comparable to conventional immunosuppressive drugs.

Nonetheless, these findings are still far from being established in medicine. Future research should surely include further investigation of exact molecular mechanisms of zinc action for a better understanding of its impact on the different components of the immune system. Although many promising results have been achieved in *in vitro* experiments, they need to be reproduced *in vivo* and the information gained has to be transferred to practical therapeutic options.

Establishing reliable methods for the assessment of a person's zinc status is necessary to allow individual supplementation and therapy and will probably lead to more consistent results in research. In this context, there are still many open questions as to what is the optimal dosage for supplementation and/or other therapeutic administration of zinc, and in which form it should occur. All in all, the influence of zinc on human health is very diverse and, as already mentioned above, becomes more and more relevant for public health. Zinc could have therapeutic applications for age-related problems, but also offers potential benefits for public health in general.

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148 Diet, immunity and inflammation

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150 Diet, immunity and inflammation

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156 Diet, immunity and inflammation

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6

The role of dietary selenium in inflammation and immunity

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Abstract: Selenium (Se) is an essential micronutrient that is important for various aspects of human health, including optimal immune responses. This chapter summarizes evidence to date regarding the effects of dietary Se on allergies and asthma, aging immunity, clinical sepsis, immunity against various pathogens and other types of inflammation and immune responses. Also presented is information pertaining to mechanisms by which Se influences inflammation and immunity, including roles for individual selenoproteins.

Key words: selenium, inflammation, immunity, selenoproteins, antioxidants.

6.1 Introduction

Selenium (Se) is an essential micronutrient that is important for various aspects of human health, including proper thyroid hormone metabolism, cardiovascular health, prevention of neurodegeneration and cancer and optimal immune responses. With a few exceptions, most populations worldwide acquire dietary Se at levels that do not result in severe deficiency or toxicity. However, there is much evidence to suggest that less overt changes in Se status may dramatically affect inflammation and immune responses (Fig. 6.1). Interestingly, various types of immune responses are not all equivalently affected by dietary Se levels. Despite strong evidence suggesting an important role for dietary Se in modulating the immune system, the mechanisms by which this essential nutrient may affect inflammation or immunity are only beginning to be understood.

The overall goals of this chapter are to explain how Se is utilized by the immune system for optimal function and to summarize evidence to date regarding the

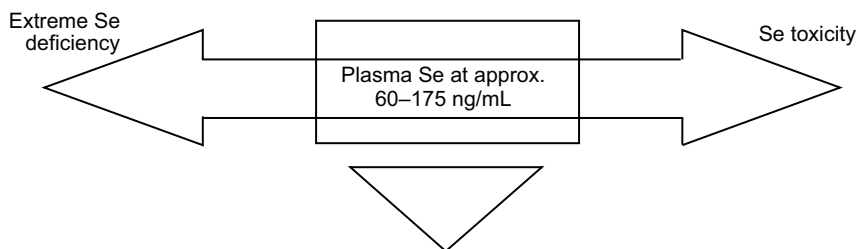


Fig. 6.1 The continuum of selenium status. Dietary Se intake that is within the 'normal' range may still affect inflammation and immunity. Less overt differences in Se status may still affect human health, particularly immune responses.

effects of dietary Se on different types of immune responses. This chapter will largely focus on information derived from human and rodent studies, as well as cell culture experiments. First, a description of the nutritional properties of Se is provided along with details on how this nutrient is metabolized and incorporated into selenoproteins, which are described in terms of their biological functions and sensitivity to changes in Se status. The next section provides details regarding how dietary Se levels affect different types of inflammatory conditions or immune responses. This includes immunity against infections with viruses, parasites and pathogenic bacteria. Next, the effects of Se levels on clinical sepsis, colitis, cancer, allergies and asthma and the aging immune system are discussed. This is followed by insights made into the mechanisms by which Se influences the immune system and comments on likely future trends pertaining to the use of Se supplementation in the modulation of inflammation and immunity.

6.2 Nutritional aspects of selenium

6.2.1 Selenium deficiency and toxicity

Dietary Se is essential in trace amounts and is attained through a wide variety of food sources including grains, vegetables, seafood, meats, dairy products and nuts (Finley, 2006). Similar to many other micronutrients, extremely high or extremely low levels of Se intake are detrimental for the health of humans as well as livestock, poultry and other organisms. Historically, the detrimental effects of Se on human health were the first to gain attention with the predominant view of Se as a lethal or carcinogenic element and early efforts were mainly focused on limiting its intake (Flohe, 2009). Chronic Se toxicity in humans results in selenosis, which is characterized by hair loss, fingernail brittleness, gastrointestinal disturbances, skin rash and abnormal functioning of the nervous system (Goldhaber, 2003). Other related toxic effects include a severe disruption of endocrine function and particularly high levels of dietary Se have been associated with diminished thyroid hormone levels and hepatotoxicity.

An appreciation for the benefits of dietary Se eventually emerged beginning in 1957 when the essentiality of dietary Se was revealed through important nutritional experiments conducted in rats (Schwarz and Foltz, 1999). While severe Se deficiency is not common in humans, some rare disorders have been identified that involve Se deficiency including Keshan disease (Li *et al.*, 1985), Kashin–Beck disease (Yang *et al.*, 1988) and myxedematous cretinism (Vanderpas *et al.*, 1990). Marginal/moderate Se deficiency can be considered a risk factor for several diseases of public health importance, but proof of cause and effect relationships will depend on a more complete understanding of basic mechanisms of action and on better analytical procedures and functional tests to determine marginal selenium status (Mertz, 1981; Muller *et al.*, 2010; Kipp *et al.*, 2012). It should be noted that Se is most likely not the only etiological factor for these disorders and co-factors such as viral infections or iodine deficiency most likely play a significant role.

6.2.2 Geography and selenium status

Average dietary intakes of Se vary between geographic regions, and this influences the nutrient intake recommended for different countries. For example, the USA has relatively high Se content in the soil and high average Se intakes, and the dietary recommendation is 55 µg per day for both men and women (Institute of Medicine, 2000). Much of Europe has lower averages of Se intake compared to the USA, and countries such as the UK recommend higher Se intakes of 75 µg per day for men and 60 µg per day for women (UK DOH, 1991). In New Zealand, low Se soil content has historically led to relatively low Se intake, but Se intake may have more recently been increased due to measures such as wheat importation and Se supplementation (Duffield *et al.*, 1999; Thomson, 2004). Recommended daily intakes for each country are based on the amount needed to maximize activity of the selenoprotein, plasma glutathione peroxidase (GPx). However, there is growing debate over the use of plasma GPx activity as a basis for setting Se requirements, and other biomarkers like plasma selenoprotein P (Sel P) have been suggested as superior biomarkers. Sel P has been shown to exhibit a better response to different dietary forms of Se and a higher plateau with increasing Se intake (Hurst *et al.*, 2010; Xia *et al.* 2010). Three major forms of Se used to supplement human and animal diets include sodium selenite, selenomethionine and high-Se yeast (which mostly consists of selenomethionine). They are metabolized differently and may have different properties pertaining to health benefits (Hatfield and Gladyshev, 2009).

6.2.3 Selenium metabolism and selenoprotein synthesis

Foods from plant and animal sources differ in Se content. The main selenocompound in plants is selenomethionine, but also present are selenate, selenite, selenocystine, dimethyl diselenide and others. Animal tissues mainly contain selenocysteine, selenomethionine, selenotrisulfides of cystine, selenate and selenite (Navarro-

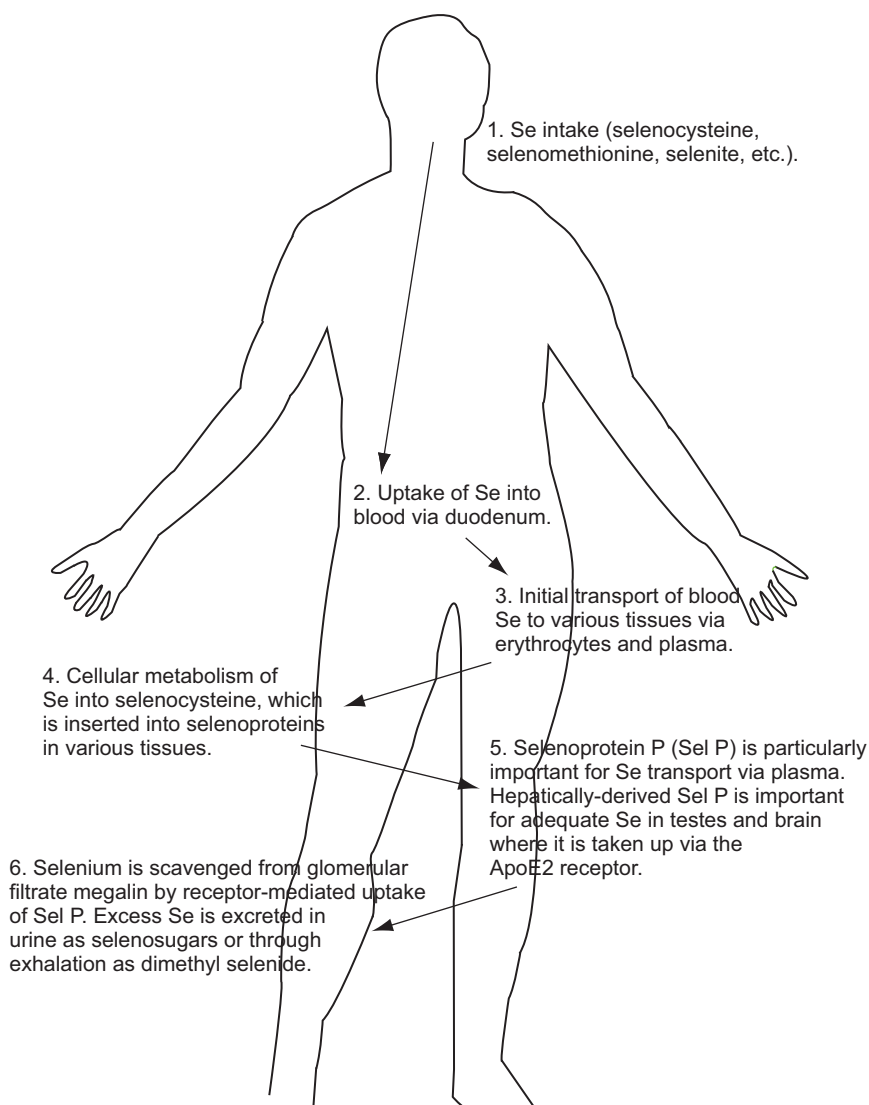


Fig. 6.2 Whole-body handling of dietary Se from ingestion to excretion.

Alarcon and Cabrera-Vique, 2008). Overall, the major form of Se ingested by humans is selenomethionine. Absorption of Se into duodenum capillaries is efficient and is not regulated. More than 90% of selenomethionine is absorbed by the same mechanism as methionine itself (Swanson *et al.*, 1991). Although less is known about selenocysteine absorption, it appears to be absorbed very well. Selenate is absorbed almost completely, but a significant fraction of it is lost in the urine before it can be incorporated into tissues. Excretion of excess Se in urine is also a mechanism by which Se levels are regulated in the body (Bugel *et al.*, 2004).

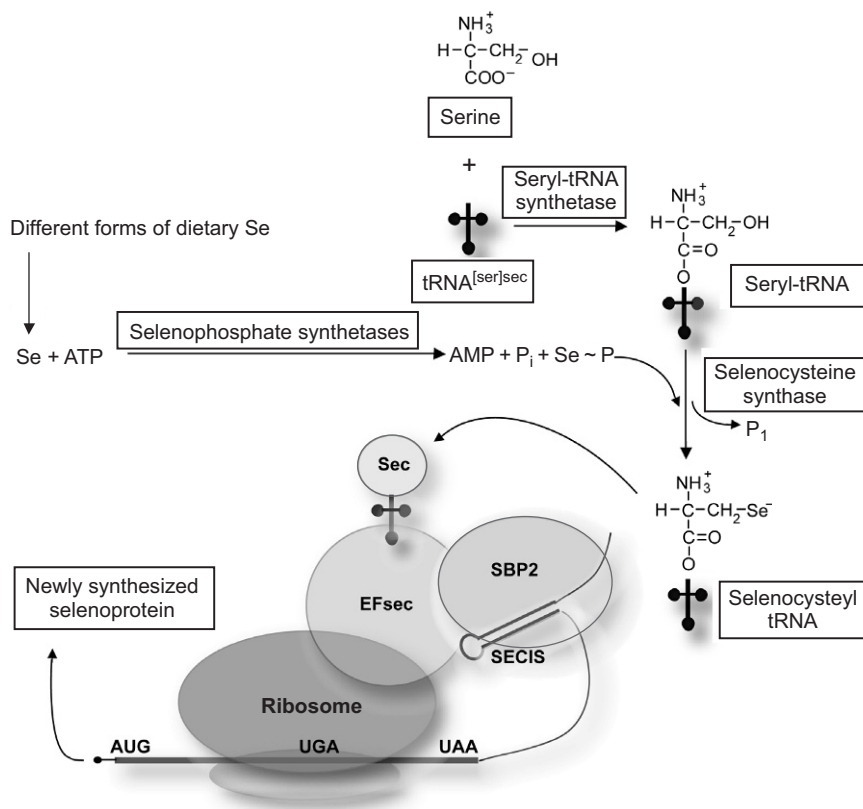


Fig. 6.3 Biochemical pathway of selenoprotein synthesis. Dietary Se is converted to selenophosphate to generate a unique tRNA, selenocysteyl tRNA, which is used by translation machinery to insert selenocysteine (Sec) during selenoprotein synthesis. AMP, adenosine monophosphate; ATP, adenosine triphosphate.

The kidneys play a key role in maintaining proper Se homeostasis by scavenging Se in glomerular filtrate via megalin receptor-mediated uptake of the key Se transporter, selenoprotein P (Sel P). The major urinary Se metabolites are selenosugars (Francesconi and Pannier, 2004). Other excreted seleno compounds such as newly identified selenoneine may protect against toxins and future studies may provide insight into the role of these compounds in Se metabolism. Figure 6.2 illustrates the overall pathway that nutritional Se takes through the body.

Many questions remain as to whether the metabolism of Se varies among different groups of people. Gender and age are factors that influence Se and selenoprotein levels in rodents (Pinto and Bartley, 1968; Suzuki *et al.*, 2005; Schomburg *et al.*, 2007). Some descriptive studies in humans have also found differences in Se-dependent health effects between men and women (Waters *et al.*, 2004). Cross-sectional analyses have provided conflicting data, with some large studies showing higher Se status in males (Galan *et al.*, 2005), while others show

higher Se status in females (Safaralizadeh *et al.*, 2007; Malling *et al.*, 2010). Whether these differences reflect different nutritional practices between geographic locations, mean age of the populations, or other factors is unclear. Overall, there is growing evidence that gender and age need to be considered when analyzing Se status or response to Se supplementation, particularly in relation to inflammation and immunity.

Within cells, most forms of Se are ultimately shuttled into a complex selenoprotein biosynthesis pathway (Allmang *et al.*, 2009), which is depicted in Fig. 6.3. Unlike other essential trace elements such as zinc and iron, Se is incorporated directly into proteins as an amino acid, selenocysteine (Sec). Selenoprotein biosynthesis is initiated by the charging of serine (Ser) onto a dedicated tRNA (tRNA^{Sec}) to generate Ser-tRNA^{Sec}. The seryl residue of Ser-tRNA^{Sec} is enzymatically phosphorylated, and then is converted to Sec-tRNA^{Sec} using monoselenophosphate as a donor of Se. The Sec-tRNA^{Sec} is used to transfer Sec into nascent selenoproteins co-translationally through a mechanism that requires several dedicated *cis* elements present in the selenoprotein mRNA (SECIS element) and protein factors that act in *trans* including SBP2 and EFsec and others (Squires and Berry, 2008). This results in recoding UGA from a stop codon to a Sec-insertion codon and the resulting protein contains the Sec amino acid, which is utilized by selenoproteins for various biological processes.

6.3 The selenoprotein family

6.3.1 Overview of selenoproteins

Dietary Se may exert effects on immunity through small molecular weight selenocompounds, but selenoproteins are believed to account for most of the biological activity of Se. Selenoproteins are defined by presence of the 21st amino acid, Sec, within the protein sequence. In 2003, innovative bioinformatic analyses of the human genome revealed a total of 25 human genes encoding selenoproteins (Kryukov *et al.*, 2003), 24 of which exist as Sec-containing proteins in mice and rats. The essentiality of selenoproteins was demonstrated in mice lacking the tRNA required for translation of all selenoproteins, which resulted in embryonic lethality (Bosl *et al.*, 1997). Because of the requirement of Sec-tRNA^{Sec} for synthesis of all selenoproteins, an effective tool for studying deletion of all selenoproteins in mice has been generated in the conditional knockout of the gene encoding Sec-tRNA^{Sec}, *trsp* (Carlson *et al.*, 2009b). While broadly classified as antioxidants, selenoproteins actually exhibit a wide range of tissue distribution, cellular locations and functions (Table 6.1). Interestingly, functions for several selenoproteins remain unclear or altogether unknown. Some of the more fully characterized subgroups of selenoproteins are described below.

6.3.2 Glutathione peroxidases

The first selenoprotein identified in mammals was glutathione peroxidase-1

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Table 6.1 Selenoproteins and their functions

Selenoprotein	Abbreviation	Function
Cytosolic glutathione peroxidase	GPx1	Peroxide reduction in the cytoplasm
Gastrointestinal glutathione peroxidase	GPx2	Peroxide reduction, mainly in the gastrointestinal tract
Plasma glutathione peroxidase	GPx3	Peroxide reduction in plasma and other extracellular fluids
Phospholipid hydroperoxide glutathione peroxidase	GPx4	Reduction of phospholipid hydroperoxides
Olfactory glutathione peroxidase	GPx6	Peroxide reduction, found in embryos and in the olfactory epithelium
Thioredoxin reductase Type I	Trxr1, TR1	Cytoplasmic thioredoxin reductase, involved in many biological pathways
Thioredoxin reductase Type II	Trxr2, TR3	Mitochondrial thioredoxin reductase, involved in many biological pathways
Thioredoxin reductase Type III	Trxr3, TR2, TGR	Thioredoxin/glutathione reductase found in mainly in testes
Deiodinase Type I	D1, DIO1	Important for systemic active thyroid hormone levels
Deiodinase Type II	D2, DIO2	Important for local active thyroid hormone levels
Deiodinase Type III	D3, DIO3	Inactivates thyroid hormone
Selenoprotein H	Sel H	Binds DNA and is involved in transcription
Selenoprotein I	Sel I, hEPT1	Possibly involved in phospholipid biosynthesis
Selenoprotein K	Sel K	Transmembrane protein localized to endoplasmic reticulum, involved in calcium mobilization
Selenoprotein M		Possibly involved in protein-folding in the ER
Selenoprotein 15	Sel M, Sep 15	Possibly involved in protein-folding in the ER
Selenoprotein N	Sel N, SEP1, SepN	Involved in RyR-related calcium mobilization from ER and potential role in early muscle formation
Selenoprotein O	Sel O	Unknown
Selenoprotein P	Sel P, SePP	Selenium transport and also functions as intracellular antioxidant in phagocytes
Selenoprotein R	Sel R, MsrB1	Functions as a methionine sulfoxide reductase
Selenoprotein S	Sel S, SEPS1, SELENOS, VIMP	Transmembrane protein involved in ER stress
Selenoprotein T	Sel T	ER protein involved in calcium mobilization
Selenoprotein V	Sel V	Testes-specific selenoprotein of unknown function
Selenoprotein W	Sel W, SEPW1	Putative antioxidant role, perhaps important in muscle growth
Selenophosphate synthetase	SPS2	Involved in synthesis of all selenoproteins, including itself

ER, endoplasmic reticulum; RyR, ryanodine receptor.

(GPx1) (Rotruck *et al.*, 1973). In addition to GPx1 (cellular GPX), other members of this subfamily include GPx2 (intestinal GPX), GPx3 (plasma GPX) and GPx4 (phospholipid GPX). While GPx1 and GPx4 are expressed in most tissues, GPx2 is expressed mainly at the epithelium of the gastrointestinal tract and GPx3 is synthesized predominately in kidney. Of these four GPx enzymes, only GPx3 is secreted into circulation for use by other tissues. GPx6 is a selenoprotein in humans (in mice, GPx6 contains Cys instead of Sec) that is localized to olfactory epithelium and embryonic tissues (Kryukov *et al.*, 2003). The GPx enzymes utilize Se at their active sites to detoxify reactive oxygen species (ROS) including hydrogen peroxide and phospholipid hydroperoxide. GPx1 and 4 are among the most abundant selenoproteins in several immune cells and tissues (Hoffmann *et al.*, 2007a; Carlson *et al.*, 2010).

6.3.3 Thioredoxin reductases

The thioredoxin reductase (Trxrd) enzymes are another well-characterized subfamily of selenoproteins that perform an essential role in the regeneration of reduced thioredoxin (Trx), which provides reducing capacity for maintaining balanced redox tone within cells (Turanov *et al.*, 2010; Lu *et al.*, 2009). The three Trxrds include cytoplasmic/nuclear Trxrd1 (also called TR1 or TrxR1) that reduces Trx1, mitochondrial Trxrd2 (also called TR3 or TxnR2) that reduces Trx2, and testes-specific thioredoxin-glutathione reductase (also called Trxrd3, TR2, TxnR3, or TGR). The essential roles of Trxrd1 and 2 during development are evident by studies in mice demonstrating that genetic deletion of either results in embryonic lethality (Conrad *et al.*, 2004; Jakupoglu *et al.*, 2005). Trxrd1 is particularly important for maintaining redox tone in immune cells through regeneration of reduced cytosolic Trx.

6.3.4 Deiodinases

The iodothyronine deiodinase family is central for thyroid hormone regulation and consists of three enzymes: types 1, 2 and 3 (DIO1, 2 and 3) (Schomburg and Kohrle, 2008). Thyroid hormone action is initiated by the activation of T4 prohormone to T3, which is carried out by DIO1 or DIO2. T4 and T3 are irreversibly inactivated in a reaction catalyzed by DIO3. All three deiodinases are expressed in a number of fetal and adult tissues, but there has been little direct correlation between thyroid hormone metabolism and immune function.

6.3.5 Selenoprotein P

Selenoprotein P (Sel P or Sepp1) is perhaps the best-characterized member of the selenoprotein family that does not belong to the above subgroups. Sel P is unique in that it contains multiple Sec residues (ten per Sel P molecule in humans and rodents). Sel P has been shown to play an important role in the transport of Se through the plasma to certain tissues, with the testes and brain particularly

dependent on Sel P for adequate Se levels (Saito and Takahashi, 2002; Hill *et al.*, 2003; Burk and Hill, 2009). Sel P is synthesized in several different tissues, but hepatically-derived Sel P serves as a key Se transporter. Hepatic Sel P is secreted into plasma, which then influences whole-body Se homeostasis (Schweizer *et al.*, 2005). Interestingly, studies in mice have shown that expression of liver Sel P is higher in females compared to males (Schomburg *et al.*, 2007). There is mounting evidence that Sel P not only transports Se but also performs crucial antioxidant functions, which are particularly important for certain immune functions as discussed in more detail below.

6.3.6 Other selenoprotein family members

What defines members of the selenoprotein family is the incorporated Sec residue, but how the different selenoproteins functionally utilize Sec is quite diverse. Some biological functions include transcriptional regulation (Sel H), phospholipid synthesis (Sel I), protein-folding (Sel M and Sep15), methionine sulfoxide reduction (Sel R), retrograde translocation of unfolded proteins from the endoplasmic reticulum (ER) (Sel K and Sel S), Ca(2+) flux modulation in immune cells (Sel K) and the biosynthesis of selenoproteins (SPS2) (Huang *et al.*, 2012b). Most of these functions are necessary for proper functioning of most tissues and cell types, including those involved in immune responses.

6.4 The effect of decreased selenium (Se) intake on selenoprotein synthesis

Under conditions of Se deficiency, not all tissues are equivalently supplied with the limited amounts Se and expression of each selenoprotein is differentially affected (Schomburg and Schweizer, 2009). This concept is often referred to as ‘the hierarchy of selenoprotein synthesis’ and must be carefully considered when investigating the effects of low Se status on immune responses or other aspects of human health. Well before elucidation of the full selenoproteome in 2003, rodent studies had shown that Se-deficient diets resulted in the loss of Se-containing proteins in most tissues, but that the Se status of the brain, testes and endocrine organs was preferentially retained (Behne and Hofer-Bosse, 1984; Behne *et al.*, 1988). Levels of certain selenoproteins such as Sel P and DIO1 are more resistant to Se deficiency compared to others like GPx1, and regulation of these levels is mainly at the level of mRNA (Hill *et al.*, 1992). There are differences even within the GPx subfamily (GPx1 through GPx4): Se deficiency leads to degradation of GPx1 and GPx3 mRNA before GPx2 and GPx4 mRNA (Weiss Sachdev and Sunde, 2001; Winkler *et al.*, 2001; Muller *et al.*, 2003). Under low Se conditions, the selenoprotein mRNAs are degraded through a process termed non-sense mediated decay (Maquat, 2001; Weiss Sachdev and Sunde, 2001), but transcriptional regulation or protein turnover must also be considered as points of regulation of selenoprotein levels. Another level of regulation involves different isoforms of

the Sec-tRNA^{Sec}, which are dependent on Se status and preferentially promotes biosynthesis of different selenoproteins (Carlson *et al.*, 2005).

6.5 Selenium and viral immunity

6.5.1 Effects on the host versus the virus

The relationship between dietary Se and various viral infections has been well investigated in animals and, to a lesser extent, in humans. Perhaps the clearest lesson learned from research on this topic is that Se-deficient hosts are very likely to experience higher susceptibility and heightened symptoms associated with a variety of viral infections. The benefits of supplementing diets with Se above adequate levels are not as clear and most likely depend on the specific virus involved. Regarding the detrimental effects of Se deficiency, there is much evidence that impaired viral clearance by a weakened immune system contributes to increased morbidity or mortality. However, in some cases low Se status in the host may also directly alter the virulence of the pathogen itself. This concept emerged mainly from studies conducted by the laboratory of Dr Melinda Beck. In these studies, Se-deficiency in mice resulted in genomic mutations in influenza and coxsackie virus that generated more virulent strains and more severe disease (Beck *et al.*, 2004). The lower Se status is thought to produce oxidative stress in infected tissues that leads to mutational changes in the viral genome (particularly for RNA viruses), giving rise to increased virulence. This has implications for human populations where viral infections occur in the context of dietary Se-deficiencies, such as Se-deplete regions of China where Keshan disease is highly endemic (Beck and Levander, 2000). Another possible example of increased virulence includes the medical mystery surrounding Kashin–Beck disease, where low Se status may be a co-factor along with potential parvovirus B19 in onset of this osteoarthritic disease of unknown etiology (Stone, 2009). However, the connection between parvovirus B19 and Kashin–Beck disease is not by any means clear. Overall, there are two separate effects of low Se status on viral infections to consider: one on the host and the other on the virus. Both of these effects may dramatically change the clinical outcome. Although virulence is an important issue, the remainder of this section will focus on the influences of Se status and selenoprotein expression on immune responses in regards to different viral infections.

6.5.2 Selenium and influenza viruses

Studies comparing Se-deficient to Se-sufficient mice infected with influenza A virus (Influenza A/Bangkok/1/79 (H3N2)) demonstrated the importance of adequate Se intake for viral clearance and recovery (Beck *et al.*, 2001). Viral titers were similar between Se-deficient and Se-sufficient hosts. However, Se deficiency appeared to alter the immune cell subsets and cytokine profiles during the course

of infection. The cytokines produced by immune cells responding to influenza infection were altered by Se status, but no mechanistic data were presented to explain these differences. Interestingly, Se status had no effect on antibody production, suggesting that Se deficiency affects cell-mediated immunity to a greater extent than humoral immunity in this model.

Se supplementation was found to be protective against H1N1 influenza infection (Yu *et al.*, 2010). In this study, mice were fed deficient (0 ppm), adequate (0.2 ppm) and supplemented (0.3–0.5 ppm) levels of Se in their diets and infected with H1N1 at a dose that resulted in 41% survival in mice fed adequate Se diets. Only 25% of mice fed low-Se diets survived, but 75% mice fed higher Se diets survived. Antiviral cytokines, tumor necrosis factor (TNF)- α and interferon (IFN)- γ , were increased with increasing dietary Se, but no indicators of adaptive immunity or humoral immune response were evaluated. Interestingly, there was no clear correlation between dietary Se and clearance of H1N1 as measured by viral titers. This is similar to the results in experiments described above for H3N2, where viral titers were not affected and suggest that the effects of Se levels on survival were more than improved viral clearance. More complete analyses of viral clearance using different doses of input virus or evaluation of different tissues over time may have revealed more robust anti-viral responses with increased Se intake. In addition, no information was obtained regarding RNA genomic mutations possibly induced by lower Se intake. Regardless, the correlation between increased dietary Se, increases in antiviral cytokines and improved resistance to influenza infection suggests that more Se intake is better for fighting these infections.

6.5.3 Selenium and poliovirus

A rare study in humans demonstrated a boost in anti-poliovirus immunity in individuals supplemented with Se (Broome *et al.*, 2004). In this study, individuals with low Se status were supplemented with either 50 or 100 μg Se daily for 15 weeks and then orally vaccinated with live, attenuated poliomyelitis vaccine and evaluated for immune responses. Se supplementation boosted T cell numbers and antiviral cytokines like IFN- γ , and led to more rapid viral clearance. Similar to the influenza studies mentioned above, humoral responses were not affected by Se intake. Also, Se supplementation minimally affected NK cell-mediated cytotoxicity (Broome *et al.*, 2004). This is in contrast to studies involving other pathogens showing that low serum Se levels were associated with low percentage of NK cells and Se supplementation increases functional properties of NK cells (Ravaglia *et al.*, 2000; Wang *et al.*, 2009a). This study was a rare demonstration of the effectiveness of Se supplementation on enhancing immunity in individuals who had an initially low Se status. This also emphasizes the importance of evaluating individuals for Se status or considering regional average Se intakes prior to beginning such studies to understand the effects of increased Se intake on health outcomes. More studies like this are needed to determine the effects of Se supplementation on immune responses in humans.

6.5.4 Selenium and HIV-1 and AIDS

Human immunodeficiency virus-1 (HIV-1) is a viral infection that both affects and is affected by host Se status. This complicates the investigation of cause and effect regarding Se intake and HIV-1 disease progression. Despite this complication, some important information has been revealed from various studies. For example, HIV-1 infection was shown to alter the expression of selenoproteins in T cells (Gladyshev *et al.*, 1999). Among HIV-1-infected individuals, lower serum Se concentrations have been associated with lower CD4⁺ T cell counts, greater HIV-1 disease progression and higher HIV-1 related mortality (Baum *et al.*, 1997; Look *et al.*, 1997; Kupka *et al.*, 2004). In addition, risk of developing mycobacterial infection in HIV-1-infected individuals was found to be higher in those individuals with serum Se lower than 135 µg/L (Shor-Posner *et al.*, 2002). Whether Se status is directly related to the ability of the immune system to fight infection or whether serum Se concentration is an indicator of overall nutritional or metabolic status has not been firmly established (Drain *et al.*, 2006). Daily Se supplementation has been shown to correlate with slower progression of HIV-1 viral burden and improved CD4⁺ T cell count (Hurwitz *et al.*, 2007). However, analyses of human studies have not shown a clear benefit of Se supplementation for HIV-1-infected individuals and confounding variables such as use of antiretroviral therapy and baseline health status make interpretation of data difficult (Pitney *et al.*, 2009). An important question is whether Se supplementation increases anti-HIV-1 immunity or the ability of infected cells to limit HIV-1 replication. This question was addressed by a study in which it was demonstrated that Trxrd1, a selenoprotein that is highly sensitive to Se status, targets the HIV-1 protein Tat and directly inhibits HIV-1 replication (Kalantari *et al.*, 2008). HIV-1 infects immune cells including T cells, macrophages and dendritic cells and causes immunosuppression, and increased Se status leading to higher levels of Trxrd1 may directly lower Tat-dependent replication in these cells. Thus, increasing Trxrd1 expression along with other selenoproteins by supplementing HIV/AIDS patients with Se may provide a potentially inexpensive therapy.

6.6 Selenium and bacterial and fungal infections

6.6.1 Direct effects of selenium on bacteria

While fungi do not express selenoproteins, analyses of bacterial genomic sequences suggest that approximately 20% of completed genomes contain selenoprotein genes (Kryukov and Gladyshev, 2004). So, does host Se status affect the bacteria themselves? Specific bacterial species may utilize host Se for their own benefit and the effect of Se supplementation for enhancing immune responses must be weighed in conjunction with this potential side effect. On the other hand, Se supplementation at high levels may have direct toxic effects on some bacterial species and this effect may synergize with boosted immunity resulting from Se supplementation. In fact, direct antimicrobial activity of different forms of Se has been demonstrated for certain bacterial infections (Yang *et al.*, 2009; Kumar *et al.*,

2010). The antibacterial effect of organic Se compounds may also provide a basis for using certain forms of Se for the inhibition of biofilms that may form on surgical wounds or implanted medical devices (Tran *et al.*, 2009). In addition, nosocomial infections involving drug-resistant bacteria are an emerging threat and Se deficiency has been associated with a variety of hospitalized patients including pediatric patients with burns and critically ill patients (Geoghegan *et al.*, 2006; Dylewski *et al.*, 2010). One must keep the direct bactericidal effects of Se in mind when evaluating studies involving anti-bacterial immunity and dietary Se.

6.6.2 The status of selenium and its effect on listeriosis and salmonellosis

Listeriosis and salmonellosis are two foodborne bacterial diseases that cause morbidity or mortality in humans. Early studies in rats and a recent study in mice have demonstrated impaired immunity in Se-deficient rodents during infection with *Listeria monocytogenes*, the main causative pathogen of listeriosis in humans (Murray and Murray, 1985; Wang *et al.*, 2009a). The impaired responses resulting from low Se intake included decreased cytokine secretion and reduced NK cytotoxicity. Also, antioxidant markers were higher in the Se-adequate mice compared to Se-deficient mice. Not all cell types apparently require adequate Se levels for antibacterial function. For example, killing of *Salmonella typhimurium* and *Staphylococcus aureus* by neutrophils was found to be unaffected by Se deficiency (Boyne *et al.*, 1984). However, the full benefits of adequate Se status during staphylococcus infection may not be realized except under conditions of high bacteremia, because only injection of high doses of bacteria produced mortality differences when comparing Se-deficient and -sufficient rats. Overall, these studies highlight the importance of maintaining adequate levels of Se intake for full immunity against bacterial pathogens. Supplementation with Se above adequate levels was not included, so no conclusions can be drawn regarding any potential for further immune enhancing or protective effects from above-adequate Se diets.

6.6.3 Selenium and tuberculosis

Tuberculosis (TB) is a bacterial disease that remains a serious health concern and a major cause of mortality worldwide, by itself or in the context of malnourished populations or HIV-1 co-infection. Se levels have been reported to be lower in TB patients compared to matched controls, and this has raised questions as to the potential benefit of Se supplementation on immunity and overall health of TB-infected patients (Kassu *et al.*, 2006; Eick *et al.*, 2009). In one study involving both HIV-1-positive and -negative patients diagnosed with pulmonary TB, supplementation with Se together with vitamins A, B complex, C and E was found to decrease the risk of TB recurrence (Villamor *et al.*, 2008). In HIV-1 patients, this may have involved improved immune responses as the supplementation increased CD3⁺ and CD4⁺ T cell counts. How much of this enhanced T cell response was due to Se versus other nutrients is unclear. A small study demonstrated promising results for

Se and vitamin E supplementation for increasing antioxidant status of TB patients, though the overall benefit of this antioxidant boost in reducing symptoms was not evident (Seyedrezazadeh *et al.*, 2008). Similar to the studies described above for salmonella infection in rodents, Se status has a clear role in regulating oxidative stress during TB infection. Given the oxidative stress in TB-infected lungs, Se supplementation could provide an inexpensive means of providing both antioxidant and immune-enhancing support in infected individuals.

6.6.4 Selenium and yeast infections

The incidence of thrush, the common enteric fungal infection caused by *Candida albicans*, has increased markedly with antibiotic therapy and in the context of AIDS. Early studies demonstrated that Se-deficient neutrophils were capable of engulfing *C. albicans*, but had significantly lower microbiocidal activity (Serfass and Ganther, 1975). Indeed, Se-deficient mice are more susceptible to *C. albicans* infection (Boyne and Arthur, 1986). The authors demonstrated an impaired ability of Se-deficient, glycogen-elicited peritoneal neutrophils to kill *C. albicans in vitro*, which was repeated in other experiments and shown to be due to reduced superoxide production, oxygen consumption and glucose utilization (Kukreja and Khan, 1994). Given that fungi do not synthesize selenoproteins, Se supplementation would conceivably not benefit their growth. Thus, Se supplementation may provide an effective dietary intervention for thrush or other fungal infections.

6.7 Selenium and immunity against parasites

6.7.1 Different parasites may utilize host selenium

Similar to infections with other types of pathogens, Se deficiency has been shown to increase susceptibility to various parasites. For example, low dietary Se in mice resulted in decreased resistance to *Heligmosomoides polygyrus* upon secondary infection (Au Yeung *et al.*, 2005). Another example is *Cryptosporidium parvum*, where immune cell-mediated resistance to infection was decreased in Se-deficient mice (Wang *et al.*, 2009b). The results of these studies support the notion that low Se status results in impaired overall host immune responses, and particularly leads to inadequate T cell proliferation and reduced cytokine production. Whether supplementing diets with Se above adequate levels would confer additional protection against infections with these parasites was not addressed, and data are particularly lacking regarding parasitic infections in humans. One study involving 977 Kenyan schoolchildren demonstrated a small but significant improvement on *Schistosoma mansoni* reinfection rates with a multivitamin supplement that included Se, but the Se status of the subjects at the beginning of the study and the relative contribution of Se in the supplement was not determined (Olsen *et al.*, 2003).

6.7.2 Selenium and trypanosomes

The influence of increasing dietary Se on the outcome of parasitic infections most likely depends on the parasite involved. This is due in part to the fact that clearance of different parasitic infections involves complex innate and adaptive immune responses over the course of the infection, depending on the lifecycle of the parasite itself. These responses may occur in different phases and different tissues. One example of the complexity of inflammation and immunity during parasitic infections is African trypanosomiasis, where a shift from classical myeloid (M1) to alternatively activated myeloid (M2) responses is important for host survival (Noel *et al.*, 2004). In *Trypanosoma*-infected mice, the development of IFN- γ -dependent M1 in the acute stage of infection restricts parasite growth by secreting molecules like TNF- α , interleukin (IL)-6, and IL-12 as well as reactive oxygen and nitrogen species (Kaushik *et al.*, 2000; Magez *et al.*, 2006). This is followed by a phase of M2 immunity, type-2 cytokine production and T regulatory cells. IL-10 plays a key role in the second phase to reduce parasitic pathogenicity and protect liver tissue during infection (Namangala *et al.*, 2001). Sel P expression is induced by IL-10 during the second phase and Sel P knockout mice are more susceptible to *Trypanosoma congolense* infection compared to wild-type controls (Bosschaerts *et al.*, 2008). Importantly, the antioxidant motif of Sel P is important for reducing oxidative stress and tissue injury, and promoting parasite clearance capacity of myeloid cells and host survival. Thus, optimal Se intake for maximizing expression of Sel P during the second phase is important for clearing this parasite and surviving infection. These results are consistent with earlier studies in mice showing that Se-deficient hosts have difficulties clearing *T. cruzi* infection and recovering from this parasitic disease (Gomez *et al.*, 2002). However, one must take into consideration that Se supplementation may also affect the pathogenic properties of the *T. cruzi* parasite itself (de Souza *et al.*, 2010).

The above example suggests that when considering Se supplementation as a prevention or treatment modality for infections with trypanosomes or other parasites, one must consider the possibility that increased Se may benefit the pathogen itself. This is further supported by data involving trypanosomes, which lack sufficient capacity to reduce oxidative stress because the parasites do not have the Se-containing GPx found in the classic GPx/GR enzymatic system (Boveris *et al.*, 1980). As a result, the parasites are at least partially dependent on the host antioxidants for survival and Se supplementation may actually benefit the parasite. This notion was supported in studies involving *T. musciuli*, a mouse-specific parasite that exhibited sustained parasitemia in Se-sufficient hosts but parasite clearance in Se-deficient hosts (Ongele *et al.*, 2002). Also, Se-containing thioredoxin and glutathione pathways differ in parasitic and free-living flatworms and canonical enzymes were specifically lost in the parasitic lineage (Otero *et al.*, 2010). This further suggests that these parasites rely on host Se-dependent antioxidant enzymes. Not all parasites lack selenoenzyme antioxidant systems and, in some parasites, non-selenoprotein antioxidant systems are very important for determining virulence. One example is *Leishmania major*, which uses ascorbate peroxidase as a major antioxidant; expression levels of this enzyme influence virulence (Pal *et al.*, 2010).

6.7.3 Selenium and intestinal parasites

A study in mice involving Se deficiency and *Heligmosomoides polygyrus* showed that low dietary Se resulted in decreased resistance to the nematodes upon secondary infection (Smith *et al.*, 2005). Because IL-4 and Th2 immunity is important for clearance of this gastrointestinal parasite, this would suggest that low Se led to lower IL-4 production. However, the decreased resistance did not appear to involve IL-4, as Se deficiency had no effect on circulating levels of this key cytokine. Importantly, route of Se treatment (e.g. intraperitoneal injection vs oral ingestion) can affect antiparasite immune responses (Pilarczyk *et al.*, 2008), adding another factor to consider in the use of Se supplementation for prevention or treatment of parasitic infections.

6.8 Selenium and inflammation related to critical illness, clinical sepsis and colitis

Studies aimed at identifying correlations between dietary Se intake and general inflammatory biomarkers such as cytokines, C-reactive protein and others have largely produced no definitive relationship (Xun *et al.*, 2010). However, examination of particular inflammatory conditions such as rheumatoid arthritis or pancreatitis suggests protection from increased Se intake (McCloy, 1998; Knekt *et al.*, 2000). Clinically relevant issues involving critical illness and Se supplementation have received much recent attention. Studies have suggested that acquired critical illness stress-induced immune suppression (CRISIS) plays a role in the development of nosocomial infection and sepsis, particularly in critically ill children (Felmet *et al.*, 2005). Se is one of several nutritional factors that may be deficient during CRISIS and may lead to stress-induced lymphopenia, a predominant M2 state, and subsequent immune suppression (Carcillo *et al.*, 2009).

In fact, a variety of inflammatory conditions in humans, such as clinical sepsis, have been associated with significantly decreased Se status (Maehira *et al.*, 2002; Hollenbach *et al.*, 2008). Inducing acute-phase response in rats by injection of lipopolysaccharide (LPS) produced significantly decreased Se in plasma and liver (Maehira *et al.*, 2002). The mechanisms by which this occurs are becoming clearer with recent studies. For example, there is evidence from studies in mice that acute-phase response to LPS causes a decrease in selenoprotein synthesis in the liver (Renko *et al.*, 2009). Interestingly, findings have revealed that Sel K is required for FcγR-induced Ca²⁺ flux and low output nitric-oxide signaling (Verma *et al.*, 2011; Huang *et al.*, 2012a) and is also a novel target of calpain (Huang *et al.*, 2012a). Importantly, cleavage is regulated dynamically by toll-like receptor (TLR)-induced calpastatin expression in macrophages, mechanisms that involve macrophage-driven inflammation and immune responses. LPS induced a downregulation of factors involved in selenoprotein synthesis, including the selenocysteine-specific elongation factor (EFsec), selenophosphate-synthetase 2 (SPS2), selenocysteine-tRNA[Ser]Sec synthase (SecS) and phosphoseryl-tRNA[Ser]Sec kinase (Pstk).

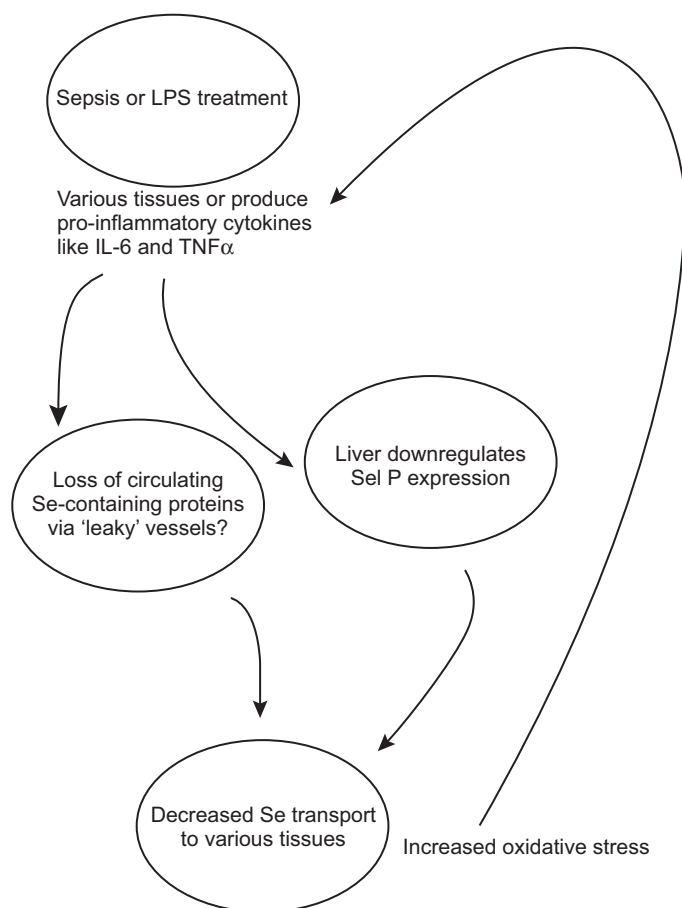


Fig. 6.4 Mechanisms by which sepsis can induce a decline in selenium status.

Along with the selenoprotein synthesis factors mentioned above, the important Se transport selenoprotein, Sel P, was also downregulated by LPS injection. The human Sel P promoter has been shown to be negatively regulated by cytokines in human hepatocytes *in vitro* (Dreher *et al.*, 1997). Thus, sepsis may trigger a pathogenic cycle in which sepsis and inflammatory cytokines decrease Sel P synthesis in the liver, which leads to lower Se levels in other tissues, which increases oxidative stress and further increases in inflammatory responses (Fig. 6.4). Intervention with Se supplementation has been shown to decrease the acute inflammatory response in LPS-injected mice as measured by TNF α , IL-6 and MCP1, but only in males (Stoedter *et al.*, 2010). This suggests a sex-specific relationship between Se status and sepsis. A different animal model of sepsis utilizing sheep demonstrated that a large-bolus injection of Se had more beneficial effects than continuous administration of Se (Wang *et al.*, 2009c). In humans, treatment of critically septic patients with Se supplementation seems to improve

clinical outcome in terms of infections and organ failure (Rinaldi *et al.*, 2009). Thus, with mechanistic data in place and preliminary data suggesting a benefit with Se supplementation, this form of nutritional intervention during clinical sepsis should be given serious consideration in clinical settings and many clinical studies are underway. Importantly, dosage and timing of Se administration must be carefully evaluated so as to not endanger these patients (Heyland, 2007).

Inflammatory bowel diseases (IBDs) such as Crohn's disease and ulcerative colitis are characterized by excessive local inflammation and tissue damage. In some cases, IBDs have been associated with decreased Se status (Ringstad *et al.*, 1993; Geerling *et al.*, 2000). This link is similar to the cyclical decline of bioavailable Se described above for sepsis patients involving increased inflammation, decreased Se status and disease progression. For example, human intestinal epithelial Caco-2 cells respond to pro-inflammatory cytokines by decreasing Sel P expression (Speckmann *et al.*, 2010). In this same study, Sel P mRNA was decreased in the colon of dextran sodium sulfate (DSS)-treated mice, an animal model of colitis. Se supplementation was shown to prevent the inflammation and improve the health of local tissue in a rat model of DSS-induced colitis (Tirosh *et al.*, 2007). This model of colitis highlights a case where localized inflammation may be mitigated by increased Se intake that bolsters the antioxidant capacity of the tissue directly involved in the inflammatory assault. It is worth noting that the effects of Se supplementation on the immune cells are less likely a factor in this model, which is quite different from allergic inflammation or autoimmune inflammatory disorders for which the influence of Se levels on immune cells is central to its effects on the inflammatory pathology.

6.9 Selenium and allergies and asthma

6.9.1 Rationale for a hypothesized relationship between selenium status and asthma

Asthma is a multifactorial inflammatory syndrome characterized by airway hyper-responsiveness, wheezing, coughing and shortness of breath (Miller, 2001; Locksley, 2010). The complex etiology of asthma may involve genetic, allergic, environmental, infectious, emotional and nutritional factors (Maddox and Schwartz, 2002). Because asthma involves increased levels of oxidative stress, Se intake has been hypothesized to play a role in the development and/or severity of this complex disease. However, significant associations between Se status and prevalence or severity of asthma have not been consistently demonstrated in human studies. This highlights both the complex etiology of human asthma and the inherent problems with correlative nutritional studies (Norton and Hoffmann, 2012). Se status can also affect the immune system and the T helper responses that drive allergic asthma (atopic asthma), which is important given that approximately half of the asthma cases in the USA and other parts of the world are believed to have an allergic component. Overall, the following sections will discuss studies in

humans and rodents related to dietary Se and asthma and how oxidative stress and immunity may be involved.

6.9.2 Epidemiological studies

A number of epidemiological studies have reported that asthma incidence, prevalence, or severity is associated with reduced Se status (Stone *et al.*, 1989; Flatt *et al.*, 1990; Hasselmark *et al.*, 1990; Shaw *et al.*, 1994; Kadrabova *et al.*, 1996; Misso *et al.*, 1996; Omland *et al.*, 2002; de Luis *et al.*, 2003; Qujeq *et al.*, 2003; Kocyigit *et al.*, 2004). However, a large, multiregional study conducted under the Global Allergy and Asthma European Network (GA²LEN) which analyzed plasma Se levels and asthma prevalence/severity from 14 centers in Europe demonstrated that Se status was not significantly associated with asthma levels in the study subjects (Burney *et al.*, 2008). Some other similar studies have also failed to confirm a link between Se levels or GPx activities and development of asthma. One study has even suggested that Se levels or GPx activities were positively associated with severity of bronchial responsiveness (Garcia-Larsen *et al.*, 2007). A large pregnancy study demonstrated that low Se levels in umbilical cord-blood were negatively associated with persistent wheeze in young children (Shaheen *et al.*, 2004). Another study found that maternal plasma Se concentration, but not GPx activity, in early pregnancy and in cord-blood was inversely associated with wheezing in early childhood (Devereux *et al.*, 2007). Overall, results of these studies are conflicting and it is difficult to clearly define the relationship between Se status and asthma. This may be due to the multifactorial etiology of this disease or variations in the study populations involved in these different studies (age of allergen exposure, atopic vs non-atopic, fluctuations in Se status over the course of disease progression, etc.).

6.9.3 Selenium supplementation for asthma patients

Similar to the epidemiological data described above, results from studies aimed at determining the effectiveness of Se supplementation for reducing the incidence or severity of asthma have also been unclear. For example, one study reported significantly decreased consumption of corticosteroids after Se supplementation with 200 µg/day for 96 weeks in corticoid-dependent asthmatics (Gazdik *et al.*, 2002). However, other studies failed to show a benefit from Se supplementation for asthmatic adults (Dunstan *et al.*, 2007; Shaheen *et al.*, 2007). Based on these findings, Se supplementation has not generally been recommended as a therapeutic modality for reducing asthma burden.

6.9.4 Mouse models of asthma

Dietary Se levels have been shown to alter the development of Th2-type immunity and ovalbumin (OVA)-induced allergic asthma in mice (Hoffmann *et al.*, 2007b, 2010). Low Se status resulted in lower Th2- or Th1-type immunity compared to

adequate Se status, while above-adequate Se status appeared to skew T helper responses away from the Th2-type responses that drive allergic asthma. Do selenoproteins play a protective role or do they promote asthma? Data from mouse studies have suggested that expression of certain selenoproteins may be induced during asthma. For example, lung GPx1 and liver Sel P were increased in OVA-challenged mice compared with controls (Hoffmann *et al.*, 2007b). A recent study reported that expression of GPx2 was increased after induction of allergic airway disease, while mice with targeted disruption of the GPx2 gene showed significantly enhanced airway inflammation compared to wild-type mice. These observations suggest that GPx2, which is more often associated with the intestinal epithelium, may play an important role in protection from allergen-induced disease (Dittrich *et al.*, 2010).

In another study, attenuation of allergen-induced eosinophilic infiltration and airway hyper-responsiveness was observed in GPx1-deficient mice compared with wild-type mice (Won *et al.*, 2010). Thus, different GPx enzymes may have opposing effects on asthma (Meyer *et al.*, 2010). This could be due to their multiple roles in both oxidative stress and immunity during the development of allergic asthma in mice. Overall, mouse feeding studies involving Se have revealed some information regarding the cross-talk between Se status, redox tone, inflammation and T helper immune responses. However, further investigation is needed to better understand how Se intake may influence the development of asthma in humans and the role of specific selenoproteins in the complex pathogenicity of allergies and asthma.

6.10 Selenium, cancer and immunity in aging

Among the potential health benefits of Se that have been studied, cancer prevention has received the most attention. Yet, the extent to which the immune system is involved in the anticancer effects of Se supplementation remains unknown. Results from the Nutritional Prevention of Cancer Trial examined the effect of high-Se yeast on cancer incidence and mortality, and results suggested a decrease in the risk of colorectal cancer, prostate cancer, lung cancer and all carcinomas combined (Clark *et al.*, 1996; Combs, 2004). In follow-up analyses to this study, supplementation with Sewas found to be associated with reduced risk of colorectal adenomas, particularly among subjects with either a low baseline Se level or among current smokers (Reid *et al.*, 2006). However, results from a subsequent study, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), failed to demonstrate evidence of any benefit against cancer for either Se or vitamin E (Lippman *et al.*, 2009). It should be noted that the design and form of Se used in the SELECT trial differed from other cancer studies (Hatfield and Gladyshev, 2009). Most of the data from these studies have focused on Se levels affecting oxidative stress, DNA mutation and the complex relationship between selenoproteins and carcinogenesis with little to no attention paid to the role of dietary Se and immune responses in preventing cancer (Hatfield *et al.*, 2009). Overall, there is a distinct

paucity of data pertaining to Se intake affecting cell-mediated clearance of cancerous tissue or any other specific aspects of anticancer immunity.

Molecular epidemiology data suggest that some selenoprotein mRNAs may be decreased in peripheral blood leukocytes (PBLs) from patients with bladder cancer compared to healthy controls (Reszka *et al.*, 2009), but any causal link was not demonstrated. An early study focusing on the effect of Se on antitumor immunity was a rare example of a human study on this issue (Kiremidjian-Schumacher and Roy, 2001). This study involved a small group of human subjects ($N = 33$) receiving either Se-supplementation (200 µg/day) or placebo and the measurement of cytotoxic T lymphocyte (CTL)-driven tumor lysis, mitogen-induced proliferation of lymphocytes and mixed lymphocyte reaction (MLR) proliferation of lymphocytes. For all three read-outs, lymphocyte performance was increased with Se supplementation. These data are similar to data from our laboratory showing enhanced signaling and proliferation in T cells with increased Se intake (Hoffmann *et al.*, 2010). In fact, there are no data suggesting that any increases in anticancer immunity that arise from increased Se intake differ from other types of cell-mediated immunity shown to benefit from increases in Se. Cell-mediated immunity would be expected to play a key role in anticancer immunity, and other data described herein suggest that higher Se intake would boost this type of immune response. Overall, there is a genuine need for more studies delineating potential effects of Se supplementation on anticancer immune responses using animal models or other mechanistic approaches.

Aging leads to a progressive decline in multiple physiological processes, including immune responses (Lesourd and Meaume, 1994; Aw *et al.*, 2007). A major issue with immunological aging is the cumulative oxidative damage to cellular components over time, and nutritional intervention is one way to prevent or limit such damage. Because Se is such a potent dietary antioxidant, it should be considered in studies focused on nutritional effects on the aging immune system. This is especially true given that elderly individuals are at risk for low Se status in certain populations (Arnaud *et al.*, 2007; Lymbury *et al.*, 2008). Se is incorporated into crucial antioxidant enzymes such as the GPx enzymes, which provide direct protection against ROS. In addition, selenoproteins such as the thioredoxin reductases, Sel R (methionine sulfoxide reductase B1), and perhaps others play key roles in reversing oxidative damage inflicted on cells. This suggests that adequate or above-adequate levels of Se may be beneficial for maintaining proper immune responses in aging individuals. A small study involving 89 men and women aged 65–80 years evaluated many nutritional markers to determine which, if any, correlated with proliferative capacity of blood lymphocytes (Wardwell *et al.*, 2008). Se was one of four nutrients found to positively correlate with proliferative capacity. Thus, Se status is thought to decline in elderly, although the data to support this are not entirely clear (Seiler, 2001).

Aging is associated with reduced IL-2 production and T cell proliferation. The role of Se in maintaining optimal T cell responses must take into consideration synergism between Se and other required nutrients. For example, vitamin E has been shown to be important for improving specific age-related T cell signaling

events in naïve CD4⁺ T cells (Marko *et al.*, 2007). Dietary Se may act in concert with vitamin E to boost aging immunity. In fact, most studies in aging individuals include several nutritional supplements in combination. Overall, most evidence suggests there are benefits of supplementation with Se and other antioxidants to prevent declining immune system function.

6.11 Mechanisms by which dietary selenium may affect inflammation and immune responses

6.11.1 Phagocytes and oxidative stress

Upon activation, phagocytic leukocytes such as macrophages and neutrophils exhibit a relatively rapid increase in ROS, referred to as an oxidative burst (Forman and Torres, 2002). In vitro studies have demonstrated effects of Se deficiency on macrophage function including poor phagocytic capacity and lower cytokine production (Hoffmann, 2007). Thus, not only is Se required for optimal activation and function of these phagocytes, but for expression of antioxidant selenoenzymes used to mitigate damage from ROS. The GPx enzymes can detoxify hydrogen peroxide while Trxrd1 is crucial for maintaining reduced thioredoxin and redox tone. Consistent with this notion, Trxrd1 mRNA and protein were shown to increase in macrophages upon LPS-stimulation (Carlson *et al.*, 2009a). In this same study, it was shown that resting macrophages lacking selenoproteins exhibited increased ROS production. As mentioned above, a study linking alternatively activated macrophages (M2) with the antioxidant activity of Sel P demonstrated a crucial role for this selenoprotein in certain innate immune responses, particularly African trypanosomiasis (Ghassabeh *et al.*, 2006; Bosschaerts *et al.*, 2008). Upregulation of Sel P in M2 during certain stages of this disease is crucial for limiting pathogenicity and oxidative damage to tissues and this raises the question of whether optimal Se intake and Sel P expression is required for resolving other diseases dependent on M2. However, many immune responses or phases of particular immune responses require strong classical macrophage (M1) responses, and whether high levels of Se intake actually skew M1 versus M2 responses one way or another must be considered.

6.11.2 ER stress-induced inflammation

One potential molecular mechanism linking Se status with inflammation was identified when single nucleotide polymorphisms in the human gene encoding selenoprotein S (Sel S or SEPS1) were correlated to serum concentrations of pro-inflammatory cytokines, e.g. IL-6, IL-1 β , and TNF- α (Curran *et al.*, 2005). The Sel S gene encodes an important factor for retrotranslocation of misfolded proteins from the ER into the cytosol for proteasomal degradation and is regulated by glucose (Gao *et al.*, 2004; Kim *et al.*, 2007). In this sense, lower levels of Sel S expression may increase inflammatory cytokines through increased ER stress. Lower levels of Se intake may produce multiple hits on ER stress by reducing

expression of proteins involved in protein folding in the ER (Sep15 and Sel M) as well as those involved in transporting misfolded proteins back to the cytoplasm for proteosomal degradation (Sel S). Interestingly, Sel S expression is increased by LPS treatment in mice, and both Se status and gender affect the influence on Sel S expression (Stoedter *et al.*, 2010).

6.11.3 T cell functions and redox tone

Human T cells respond to Se supplementation by increasing mRNA corresponding to genes encoding proteins involved in protein biosynthesis (Pagmantidis *et al.*, 2008). This upregulation in biosynthesis machinery in turn may be used to increase production of signaling molecules and other proteins that mediate activation and proliferation. For example, supplementation of C57BL/6 mice with Se resulted in increased expression of the IL-2 receptor α subunit (CD25) and increased proliferative capacity of lymphocytes (Roy *et al.*, 1993). This was later repeated in mice and shown to depend on redox status (Hoffmann *et al.*, 2007b, 2010). Increases in dietary Se in mice were shown to boost signaling strength during T cell receptor (TCR)-induced activation (Hoffmann *et al.*, 2010). TCR signaling in CD4⁺ T cells was enhanced with Se intake, leading to increased calcium mobilization, stronger oxidative burst and increased translocation of nuclear factor of activated T cells (NFAT).

Mouse models involving low dietary Se and genetic deletion of selenoproteins in T cells have suggested that this family of proteins is required for reducing oxidative stress and controlling redox tone in this immune cell (Shrimali *et al.*, 2008; Huang *et al.*, 2012b). This is in agreement with data showing that GPx1 is the most abundant selenoprotein mRNA detected in lymph node and spleen T cells (Carlson *et al.*, 2010). Interestingly, Sep15 mRNA is highly abundant in T cells as well. Given its role in folding and maturation of a restricted group of N-glycosylated proteins in the ER (Labunskyy *et al.*, 2009), Sep15 may also be an important mechanism by which dietary Se affects T cell function. Overall, redox tone in T helper cells that are activated by antigen presenting cells appears to be a critical step at which Se may influence the type and strength of immune responses that arise upon antigen challenge (Fig. 6.5).

6.11.4 B cell function and antibody production

In a study described above, Se supplementation was shown to increase antipoliiovirus immunity in regards to several aspects of cell-mediated immunity (Broome *et al.*, 2004). However, antibody titers to poliovirus were not affected by levels of Se intake, suggesting plasma B cell production of IgG was not affected by Se supplementation. Rodent studies described above involving influenza infection also showed no significant effect of increasing dietary Se on humoral responses. A very small study involving 11 men suggested that antibody titers against diphtheria vaccine, but not against influenza A or B, were increased after reinoculation in Se-supplemented versus non-supplemented individuals (Hawkes *et al.*, 2001). This

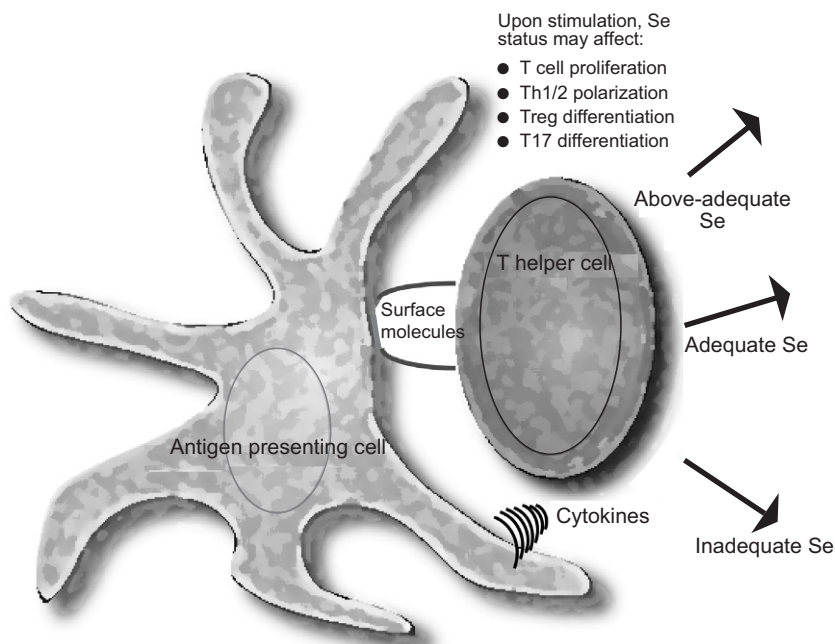


Fig. 6.5 Effects of Se status on T helper cell activation, proliferation and differentiation. Naïve T helper cells are activated by antigen presenting cells such as dendritic cells and macrophages through cell-to-cell contact and cytokine secretion. Se status can affect redox tone in both antigen presenting cells and responding T helper cells and may dramatically affect the type and strength of immune response that arises.

suggests that Se levels may affect antibody production in a pathogen-dependent manner. Of course, antibody titers are not influenced by B cell function alone, with T helper cells playing a crucial role as well. Thus, it is difficult to uncouple B cell function from T helper cell function when using humoral responses as an experimental outcome.

6.12 Conclusion and future trends

Initiation and regulation of inflammation and immune responses involve many different cell types and coordinated interactions that take place in a time- and spatial-dependent manner (Fig. 6.6). Dietary Se may impinge upon one or more of the steps involved in these processes, adding to the varied effects of dietary Se observed between individuals and between different types of inflammation and immune responses. Overall, there is much evidence that poor Se intake impairs the immune system leading to an ineffective response to infections and other challenges, and the benefits of increasing Se intake to adequate levels through supplementation are clear. Further increasing Se intake from adequate to above-adequate levels may result in additional immune enhancement, although more

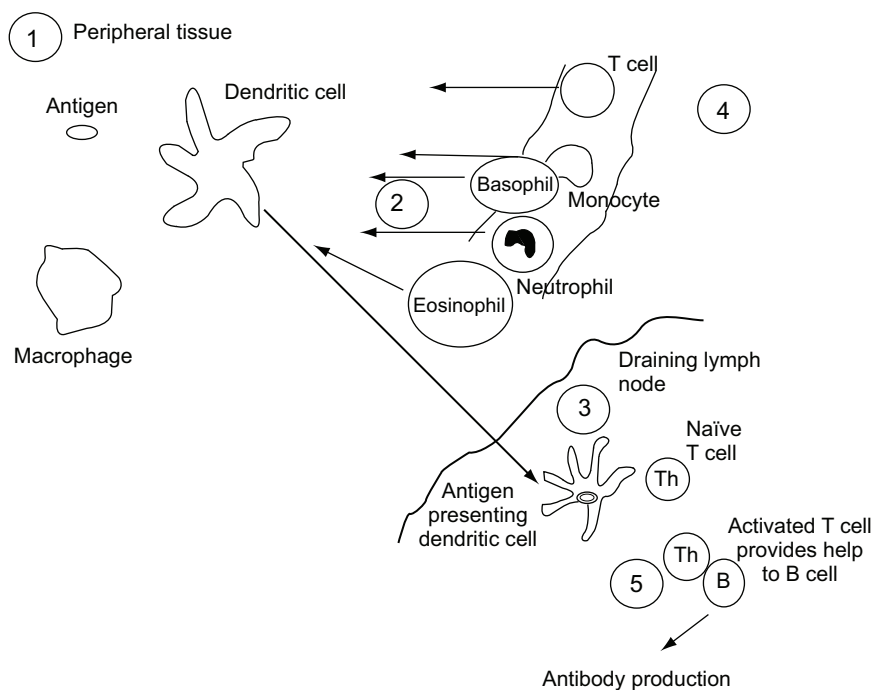


Fig. 6.6 Dietary Se may impinge upon inflammation and immunity at one or more steps. 1, Inflammation is initiated in various tissues when innate immune cells such as macrophages and dendritic cells encounter and phagocytose antigens. 2, Soluble mediators secreted from tissues result in infiltration of various leukocytes including neutrophils, monocytes and others. 3, Matured dendritic cells migrate via lymphatics to lymph nodes where they present antigen in the context of major histocompatibility (MHC) to naïve T helper cells and cytotoxic T cells. T helper cells become activated, proliferate and differentiate. 4, Activated T cells migrate via blood to inflamed tissues to provide help clearing antigen. 5, T helper cells provide 'help' to B cells for B cell activation, proliferation and differentiation and antibody is generated.

research is required to confirm enhanced immunity with attention paid to potential side effects associated with supraphysiologic intakes of Se. There is emerging evidence that high Se intake may promote development of Type 2 diabetes, but whether this is a true effect of Se supplementation remains controversial (Dunn *et al.*, 2010; Stranges *et al.*, 2010). A Cochrane review of controlled trials did not support increased risk for Type 2 diabetes with Se supplementation (Rees *et al.*, 2013), and this issue must be clarified before Se supplementation is recommended for prevention or therapy. This same review did not support a role for Se supplementation to promote cardiovascular health (Rees *et al.*, 2013). Heart inflammation in the context of Se status is important given that both Se deficiency and modest Se supplementation lead to a similar phenotype of abnormal myocardial matrix remodeling and dysfunction in the normal heart in mice, indicating it needs to be taken into account while optimizing Se status for prevention and treatment of heart failure (Metes-Kosik *et al.*, 2012).

In lieu of recommending Se supplementation for the general public for improving overall immunity or reducing chronic inflammation, future studies may lead to the use of this supplement for periodic treatment of specific disorders. For example, Se supplementation may provide an inexpensive and effective means of reversing the impaired immunity associated with aging and the immunosuppression associated with cancer and its treatment, and with HIV/AIDS. It also is clear from studies described above that Se supplementation in critically ill patients or during cases of septic shock may significantly improve the condition of the patient. Novel uses for Se supplementation may include skewing immunity away from Th2-type immune responses that drive asthma while boosting Th1 responses required for antiviral and anticancer immunity. With this in mind, combining Se supplementation with other treatments such as specific immunotherapy that is used to reduce allergies and allergic asthma may enhance the effect of these treatments by deviating responses away from Th2-type responses. Importantly, the use of Se supplementation as a means of modulating immunity and inflammation in the future will depend on a better understanding of the mechanisms by which this micronutrient affects immune responses and inflammatory processes.

6.13 Sources of further information and advice

- *Selenium: Its molecular biology and role in human health* (2nd edn), DL Hatfield, MJ Berry, and N Gladyshev (eds). New York: Springer.
- United States Department of Agriculture, Agricultural Research Service Nutrient Data Laboratory Home Page http://www.ars.usda.gov/main/site_main.htm?modecode=12-35-45-00
- National Institutes of Health, Office of Dietary Supplements Selenium Fact Sheet <http://ods.od.nih.gov/factsheets/Selenium-HealthProfessional/>

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The role of dietary selenium in inflammation and immunity 191

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7

The role of iron in immunity and inflammation: implications for the response to infection

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Abstract: Iron is required for many biochemical reactions of immune and non-immune cells, and pathogens. Iron deficiency remains a worldwide public health problem in children and women of childbearing age. Iron overload is common in individuals with genetic disorders of proteins involved in iron metabolism. The influence of iron homeostasis on the immune function and risk of infection has been recognized since the 1970s. Recent discoveries have shed light on the various proteins that modulate iron metabolism and their role in immunity. This chapter summarizes our current knowledge on the mechanisms by which iron dysregulation alters the immune response and the conflicting results relating to its effect on risk of infection.

Key words: iron deficiency, iron overload, cytokines, T and B lymphocytes, macrophages, infection, cell-mediated immunity, ferroportin-1.

7.1 Introduction

Iron is the fourth most common element in the Earth's crust and is one of the most studied nutrients in human health. Iron exists in two main ionizable forms: ferric (Fe^{3+}) and ferrous (Fe^{2+}). The ease of oxidation and reduction of iron makes it a unique trace element for many cellular redox reactions. Iron is required by virtually all living cells for many biochemical reactions including the metabolism of amino

acids, proteins, lipids, and carbohydrates, and cell proliferation (Cazzalo *et al.*, 1990). In spite of the knowledge of the role of iron in human health for many centuries, the importance of iron in immunity was first recognized only in the 1970s. This field evolved from clinical observations of an association between iron deficiency and infection (Oppenheimer *et al.*, 1986). It was later shown that some immune responses were altered by iron deficiency (reviewed by Oppenheimer, 2001; Beard, 2001). This chapter summarizes the current knowledge of the proteins that modulate iron metabolism and immunity, the effects of iron deficiency and iron overload on immunity, the mechanisms by which iron regulates the immune response and the implications on risk of infection.

7.1.1 Assessment of iron status in the human body

Adult men and women have approximately 3.5 g and 2.3 g of total body iron, respectively. This iron is distributed in various compartments: roughly 65% and 75% circulates in blood bound to hemoglobin, 22% and 12% is stored in the liver in the form of ferritin and hemosiderin, and 2.9% and 4.6% is bound to various enzymes, in men and women, respectively. In both genders, about 10% of iron is associated with myoglobin, an oxygen-binding protein found in muscle, and only 0.1% in men and 1.3% in women circulate in blood bound to transferrin.

Iron status is evaluated by three methods: clinical evaluation, hematological and biochemical laboratory tests, and therapeutic iron trials. The four common blood indexes that distinguish iron deficiency from normal status and iron overload are serum ferritin concentration, hemoglobin concentration, serum or soluble transferrin receptor concentration, and transferrin saturation. Pure iron deficiency differs from normal iron status by reduced blood levels of hemoglobin, transferrin saturation below the levels defined for age and gender, serum ferritin concentration less than 12 µg/l, and soluble transferrin receptor concentration above 8.5 mg/l (Cook and Skikne, 1989; Kuvibidila *et al.*, 1994a; Suominen *et al.*, 1997; WHO/CDC, 2007). In the presence of infection and inflammation, serum ferritin is usually within the normal range (>12 µg/l but <100 µg/l) and iron deficiency can only be ruled out by parallel measurement of soluble transferrin receptor and at least two acute phase proteins – C-reactive protein and α -1-acid glycoprotein (Kuvibidila *et al.*, 1994a,b; Beard *et al.*, 2006; WHO/CDC, 2007). Groups at risk for iron deficiency include infants, young children, and women of childbearing age. The elderly may be at risk of iron deficiency, especially if they have diseases that affect gut integrity and therefore impair iron absorption, bleeding disorders (e.g. ulcer), excessive intake of antacid, or inadequate dietary intake. Iron deficiency in infants combined with immaturity of gut immunity or iron deficiency in the elderly combined with immune senescence may increase the risk of infection.

Iron overload differs from normal iron status by increased serum ferritin concentration (>200 µg/l in men and >150 µg/l in women) and transferrin saturation >50% (WHO, 2007). Soluble transferrin receptor concentration is in general within the normal range or is slightly decreased below 3 mg/l (Khumalo *et al.*, 1998; WHO/CDC, 2007). Groups at risk for iron overload include individuals who receive

frequent blood transfusions, such as those with β -thalassemia or sickle-cell disease, patients with renal disease who receive medicinal iron due to impaired erythropoiesis, people with idiopathic hemochromatosis and the Bantu of Southern Africa who frequently consume traditional beer which is very rich in iron.

7.1.2 Iron absorption and transport proteins

The absorption of dietary non-heme and heme iron takes place in enterocytes mainly of the duodenum and the upper jejunum. In general, the amount of dietary iron that is absorbed from the diet equals losses, and is of the order of 1–2 mg/day (Beard, 2006). The reason for this tight regulation is that iron is a very potent pro-oxidant and, as such, in its free form, it promotes the formation of free radicals, which are very detrimental to cell membranes and intracellular macromolecules.

Since the mid-1990s, our understanding of the mechanisms and/or proteins involved in iron absorption has significantly increased. Most dietary iron that enters the human gut lumen is in the ferric (Fe^{3+}) form (from plant foods), which is poorly absorbed. We now know that the ferric iron is reduced to ferrous iron (Fe^{2+}) by a ferric reductase, called duodenum cytochrome B (DCytB) at the apical membrane of enterocytes prior to absorption (Zhang and Ennes, 2009). Besides DCytB, there are several other proteins that play a crucial role in iron homeostasis and modulate immunity (Porto and De Sousa 2007; Nadadur *et al.*, 2008) (Table 7.1):

- DMT1 (divalent metal transporter-1) – also called IREP2 (iron regulatory element protein-2) or NRAMP2 (natural resistance antimacrophage protein-2) – which transfers iron from the lumen to the cytoplasm of enterocytes. It also works as a chaperone within the cytoplasm.
- Ferroportin-1 – also called IREP1 – is the only known iron exporter from enterocytes and macrophages.
- Hephaestin is a copper-containing protein involved in the oxidation of ferrous iron exported by ferroportin-1 from enterocytes before it is bound to transferrin.
- Hephcidin is an iron-sensing protein produced by hepatocytes. It regulates the amount of iron released from enterocytes through ferroportin-1 to transferrin, and hence into the bloodstream. Iron deficiency reduces its expression and iron overload increases it.
- Heme carrier protein-1 (Hcp1) is a protein expressed in enterocytes and macrophages and involved in heme-iron absorption. It is increased during iron deficiency and reduced by bacterial lipopolysaccharides (LPS) (Krijt *et al.*, 2006).
- Heme-oxygenase 1, which is abundant in enterocytes and macrophages, degrades heme leading to iron release. It is reduced by iron deficiency and LPS, but increased by iron (overload) and anti-inflammatory cytokines [interleukin-6 (IL-6) and IL-10] (Ricchetti *et al.*, 2004).
- Ceruloplasmin, a ferroxidase copper-containing protein, is involved in the release of iron from hepatocytes by oxidizing ferrous to ferric iron so that it will be bound to transferrin.

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Table 7.1 Proteins involved in iron homeostasis (absorption, release, transport and storage) or that are responsive to iron status and their roles in immune function

Protein	Location/origin	Function	Response to iron deficiency	Response to iron overload	Role in immune function	Response during infection/inflammation
DCytB	Intestine	Ferri-reductase: reduction of Fe ³⁺ to Fe ²⁺ before absorption via DMT1	↑ (Barisani <i>et al.</i> , 2004)	↓	No direct role	Expected ↓ to limit iron absorption
DMT1 (NRAMP2; IREP-2)	Enterocytes, macrophages and hepatocytes	Fe ²⁺ uptake; Fe ²⁺ trafficking in the cytosol	↑ (Barisani <i>et al.</i> , 2004)	↓	Deletion will reduce ferrous iron absorption also impair MΦ function	↑ by TNF-α and IFN-γ
Ferritin	Liver, bone marrow, muscle, plasma; macrophages and T lymphocytes	Iron storage	↓	↑	No direct role	↑
Ferroportin-1	Enterocytes and macrophages	Fe ²⁺ release	↑ (Barisani <i>et al.</i> (2004)	↓	↓ NO production in ferroportin-1 over-expression	↑ in peritoneal and lung MΦ; ↓ bone marrow MΦ (Kristopher <i>et al.</i> , 2008)
Ceruloplasmin	Plasma	Copper-containing protein that oxidizes Fe ²⁺ to Fe ³⁺ and facilitates Fe release from hepatocytes and macrophages	↔	↔	Antioxidant; ↑ Ab production ↑ T cell proliferation ↑ IL-1 secretion (Kojima <i>et al.</i> , 1986)	↑
Hephaestin	Enterocytes	Copper-containing ferroxidase – oxidizes Fe ²⁺ to Fe ³⁺ iron before uptake by transferrin	↑ (Barisani <i>et al.</i> , 2004)	↓ (Barisani <i>et al.</i> , 2004)	?	? (Expected to ↓ iron release to blood-stream)

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Hepcidin	Produced by hepatocytes (and spleen cells), found in plasma	Regulates iron release from enterocytes and macrophages by interaction with ferroportin-1	↓	↑	?	↑ (Lee <i>et al.</i> , 2005)
Lactoferrin	Produced by neutrophils; found in milk, oral cavity, nasal secretions, intestinal fluids, tears, respiratory tract fluids, other body fluids	Fe transport and chelation	↓ ↔	↑ ↔	Antiviral, antibacterial, antifungal activities ↑ total leukocytes, CD3 ⁺ , CD4 ⁺ , CD8 ⁺ and lymphocyte proliferation; ↑ secretion of IL-β, IL-12, IL-18 and DTH; secretion of IgA, IgG, IgM; ↑ differentiation of T helper cells; detected on activated T and B cells, monocytes, intestinal brush border cells, platelets and neoplastic cells (Actor <i>et al.</i> , 2009)	↑ (Adlerova <i>et al.</i> , 2008)
Transferrin	Plasma	Fe transport	↑	↓	Required for clonal expansion of immune cells, and for T- and non-T-cell immune responses	↓
Transferrin receptor 1	Immune and non-immune cells	Fe delivery to cells	↑	↓	Required for iron uptake by activated lymphocytes, monocytes/macrophages, NK cells, neutrophils	↓

(Continued)

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Table 7.1 Continued

Protein	Location/origin	Function	Response to iron deficiency	Response to iron overload	Role in immune function	Response during infection/inflammation
β2-micro-globulin	All cells	Interacts with HFE, deficit leads to iron overload	?	?	Required for major histoincompatibility-class 1 expression which is required for which peptide presentation	
IL-1	Secreted by immune and non-immune (adipocytes) cells	Blocks iron absorption via hepcidin, inflammatory cytokine	↓	↑	Involved in immune and inflammatory responses; promotes iron sequestration in macrophages and liver during infection	↑
IL-6	Secreted by immune and non-immune (fibroblasts) cells; found in plasma	Blocks iron absorption via hepcidin, inflammatory cytokine	↓	↑	Involved in immune and inflammatory responses; promotes iron sequestration in macrophages and the liver during infection	↑
IFN-γ	Secreted by T and NK cells; found in plasma	Host defense	↓	↑	Involved in immune and inflammatory responses; induces iron uptake by macrophages	↑

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TNF- α	Secreted by immune cells and non-immune (adipocytes) cells; found in plasma	Blocks iron absorption and release – thus depriving it to pathogens; inflammatory cytokine	↓	↑	Involved in immune and inflammatory responses; anti-pathogen	
Hcp1	Enterocytes	Absorption of heme-iron	↑, ↔ (Krijt <i>et al.</i> , 2006)	?	No direct role	↓ by LPS (Krijt <i>et al.</i> , 2006)
Heme-oxygenase 1	Abundant in enterocytes; also present in macrophages	Degradation of heme	↓	↑	Released iron may modulate immune cell function, anti-inflammatory	↑ by IL-10 and IL-6; ↓ by LPS (Ricchetti <i>et al.</i> , 2004)
HIF- α	Ubiquitous, immune cells, specifically lymphocytes	Induction of erythropoietin and erythropoiesis	↑, ↔	↓	Regulates gene expression of growth factors (e.g. insulin-like growth factor) proliferation; induction of apoptosis via caspase	↑ (because of decreased cell Fe)
HIF-2 α	Macrophages, vascular endothelial cells	Promotes iron absorption			Induces macrophage pro-inflammatory and chemokine production	
NF κ B	Lymphocytes, monocytes, macrophages, endothelial cells	Transcription factor that regulates inflammatory gene expression	↓	↑	Induces cytokine and other inflammatory gene expression	↑
Inducible nitric oxide synthase (iNOS)	Immune cells, specifically macrophages and endothelial cells	Inducible enzyme, that produces NO	↑	↓	Antimicrobial	↓

DCyB = duodenal cytochrome b; DMT1 = divalent metal transport-1; Hcp1 = heme career protein-1; HIF- α = hypoxia inducible factor- α ; IREP-2 = iron regulatory protein-2; NRAMP2 = natural resistance antimacrophage protein-2; ↑ = increased; ↓ = decreased; ↔ = no change; ? = information not available.

- Transferrin is involved in iron transport in blood and transfer to various tissues.
- Transferrin receptor-1 is a cell membrane-associated protein through which iron is delivered into the cells. It is specifically expressed in activated immune cells (ex. macrophages, T cells, B cells).

Ferritin plays a role in iron storage rather than transport or absorption. β 2-microglobulin plays an indirect role in iron homeostasis through its interaction with HFE (high iron or high FE) – the hemochromatosis gene product that is involved in iron overload. Although HFE is expressed in many tissues, its expression is stronger in the intestine and the liver (hepatocytes and Kupffer cells) than other cell types (Cecic, 2005). Mutation in β 2-microglobulin with intact HFE, or mutation in HFE with intact β 2-microglobulin, leads to iron overload. Other proteins that modulate iron homeostasis include transferrin receptor 2 (mostly found in enterocytes, hepatocytes and erythrocytes) and hemojuvelin, which is present in several tissues (liver, heart, skeletal muscle, colon, pancreas, brain, and kidney esophagus (Cecic, 2005). Both these proteins interact with HFE and regulate iron absorption. Another set of proteins that need to be mentioned is the hypoxia inducible factor α -1 (HIF-1 α) and HIF-2 α . While HIF-1 α is ubiquitously expressed, HIF-2 α is expressed in macrophages and vascular endothelial cells (Hu *et al.*, 2003). HIF-1 α induces erythropoiesis and HIF-2 α promotes iron absorption (Mastrogiannaki *et al.*, 2009).

7.1.3 The roles of various proteins associated with iron homeostasis in immunity

The direct and indirect roles of the various proteins related to iron homeostasis in immunity are summarized in Table 7.1. Not only are many of these proteins altered by iron deficiency/overload, but their altered expression also modulates immune functions (Darshan *et al.*, 2010). For example, macrophages obtained from HFE knockout mice secrete lower amounts of tumor necrosis factor (TNF)- α and IL-6 than those obtained from wild-type mice (Wang *et al.*, 2009). Iron chelators (which mimic iron deficiency) in wild-type mice have a similar effect. The decreased cytokine secretion is due to lack of expression of Toll-like receptor 4 (TLR4) and activation of nuclear factor kappa B (NFkB) (Wang *et al.*, 2009). Since the lack of HFE gene is usually associated with upregulation of ferroportin-1, which increases iron efflux, and hence creates a state of iron depletion in the macrophages, the reduced cytokine secretion could be directly linked to lack of iron. In macrophages and dendritic cells, TLR4 is a ligand for LPS and the interaction of TLR4 and LPS upregulates cytokine gene and protein expression, often via NFkB activation (Fukata and Abreu, 2007). Low expression of TLR4 in rodents leads to reduced splenic natural killer (NK) cell cytotoxicity, plasma levels of IL-12, IL-23, and IL-17, and these effects can increase the risk of infection (Happel *et al.*, 2003; Vogel *et al.*, 2005; Gerold *et al.*, 2007).

Another protein that is regulated by iron and that modulates cytokine gene expression is HIF-1 α . At normal iron status, HIF-1 α is propyl-hydroxylated by an

iron-dependent enzyme (propyl-hydroxylase) and is degraded by proteasomes (Cherayil, 2010). During iron deprivation, the activity of hydroxylase is low, so that HIF-1 α remains intact and induces gene expression of various cytokines including those with antimicrobial properties. HIF-2 α , a factor expressed in macrophages, induces the expression of pro-inflammatory cytokines (IL-1 β , IL-12, TNF- α) and interferon-gamma (IFN- γ) and decreases IL-10 expression (Mastrogiannaki *et al.*, 2009; Imtiyaz *et al.*, 2010).

Iron status also affects NF κ B activation. At normal and elevated intracellular iron levels, NF κ B activation is increased via high production of reactive oxygen species and this, in turn, leads to increased cytokine gene expression and cytokine secretion (Bubici *et al.*, 2006; Vallabhapurapu and Karin, 2009). During iron deficiency, the production of reactive oxygen species is reduced, and so is NF κ B activation and cytokine secretion. Thus, the defense against infectious agents is compromised. In contrast to NF κ B, iron chelation in macrophages upregulates the secretion of the antimicrobial nitric oxide (NO), perhaps to prevent full-blown infection (Cherayil, 2010).

During intracellular infection of monocytes/macrophages by *Mycobacterium tuberculosis*, ferroportin-1 expression is increased, and intracellular iron is decreased since iron is more efficiently pumped out of the cells (Johnson *et al.*, 2010). Such a strategy limits iron availability to intracellular pathogens. However, it may also be detrimental since the production of reactive oxygen species that are required for pathogen killing is reduced (Fang, 2004). Lactoferrin, the iron-transport protein in milk and many other body fluids (tears, nasal secretions, etc.), has antiviral and antibacterial properties. Furthermore, at least in weanling piglets, lactoferrin promotes lymphocyte proliferation, progression of cells through the cell cycle, immunoglobulin secretion, and the differentiation of naïve T cells (Shan *et al.*, 2007; Lee *et al.*, 2009). Apo-lactoferrin has no immunoregulatory properties. Iron deficiency severe enough to lower iron-lactoferrin (and iron overload high enough to fully saturate lactoferrin) would be expected to alter lactoferrin's immunomodulatory properties. Lactoferrin is increased by inflammation. Blockade of transferrin receptor-1 by antibodies reduces lymphocyte proliferation and the functions of lymphocytes, monocytes, macrophages, and NK cells due to impaired iron-dependent enzymes (Bayer *et al.*, 1998).

7.2 The effects of iron deficiency on immunity

7.2.1 Iron deficiency and T-cell function

Resting T cells do not express transferrin receptors on their cell surface and therefore either their iron requirements are very low or they take up iron by an unknown transferrin receptor-independent pathway. Upon activation, T cells express surface transferrin receptors while in the G0/G1 phase of the cell cycle before the initiation of DNA synthesis (Neckers and Cossman, 1983; Artac *et al.*, 2006). The increase in transferrin receptor expression is believed to ensure sufficient iron to support the activity of ribonucleotide reductase, the rate-limiting

enzyme in the cell proliferation pathway (required for the biosynthesis of deoxyribonucleotides).

In recent years, the role of iron in T lymphocyte proliferation beyond ribonucleotide reductase has also been investigated. Iron is required for the expression of certain cell cycle regulatory proteins – cyclins and cyclin-dependent kinases – that regulate the passage from G0/G1 (G = gap, resting phase) to G1, from G1 to S (DNA synthesis phase), and from S to G2/M (M = mitosis). In quiescent lymphocytes, cyclin A and B mRNA, cyclin-dependent kinase-2, and cyclin-dependent kinase-4 are very low (Lucas *et al.*, 1995). However, once cells enter the G1 phase, the expression of these proteins significantly increases. Iron chelation impairs the *expression* of these cyclins and associated kinases. Since cyclin A triggers the entry of cells into the S phase, we can speculate that reduced expression of cyclin A may in part explain impaired lymphocyte proliferation associated with iron deficiency (Kuvibidila *et al.*, 2001a; see below). To date though, no studies have been conducted on the expression of cyclins in lymphocytes from iron-deficient human subjects.

There are many cell-mediated immune responses that have been investigated in iron-deficient subjects and laboratory animals (Table 7.2). In humans, most studies suggest that iron deficiency resulting from dietary restriction reduces the proportion of T lymphocytes in blood; however, the absolute number of T cells is either unaltered or reduced (Ekiz *et al.*, 2005; Mullick *et al.*, 2006; Attia *et al.*, 2009). Iron deficiency resulting from blood loss does not reduce the proportion of T cells in blood, but it decreases the total T-cell numbers (Santos and Falcao, 1990). The discrepancy between the two types of iron deficiency is very likely related to the time required for the development of iron deficiency by dietary restriction as compared with blood loss and T-cell differentiation. In iron-deficient humans, the absolute numbers of CD4⁺ and CD8⁺ T cells are either decreased or normal (Attia *et al.*, 2003, Mullick *et al.*, 2006, Tang *et al.*, 2006). In mice, iron deficiency reduces the proportion of total T cells, helper T cells, cytotoxic/suppressor T-cells and the percentage of CD28⁺ in the spleen, but not the ratio of helper to cytotoxic T cells (Kuvibidila *et al.*, 1990; Kuvibidila and Porretta, 2003).

Iron deficiency induces thymus atrophy in mice but does not affect the proportion of total T cells, helper T cells and cytotoxic/suppressor T cells or the ratio of helper to cytotoxic/suppressor T cells in the thymus (Kuvibidila *et al.*, 1990). Thymus atrophy may in part explain the trend of decreased T lymphocyte number during iron deficiency. The mechanisms of thymus atrophy are unclear but could be due to reduced thymocyte proliferation and/or increased apoptosis (Kuvibidila *et al.*, 2001b). Thymus atrophy in iron-deficient human subjects has not been investigated. In contrast to thymus atrophy, iron deficiency in laboratory mice induces splenomegaly – which could be related to ingested abnormal red blood cells by macrophages. Splenomegaly in rodents is not always associated with increased absolute lymphocyte numbers (Kuvibidila *et al.*, 1982, 1990).

Iron deficiency in humans and laboratory animals induces anergy and impairs lymphocyte proliferative responses to mitogens and antigens (Kuvibidila *et al.*, 1981, 1983a, 1998; Artac *et al.*, 2006; and reviewed by Oppenheimer, 2001).

Table 7.2 Effects of iron status on aspects of cell-mediated immunity

	Effect of iron deficiency	Effect of iron repletion	Effect of iron overload
Thymus weight (mg/g body weight)	↓	↑	?
Spleen weight (mg/g body weight)	↑	↓	↔
Blood mononuclear cell proliferation	↓	↑	↓
Spleen T cell proliferation	↓	↑	?
Thymocyte proliferation	↓, ↔	?	?
% of total T lymphocytes	↓↔	↑↔	↓↔
% of CD4 ⁺ cells	↓↔	↑↔	↓↔
% of CD8 ⁺ cells and/or activity	↓↔	↔	↑
CD4 ⁺ :CD8 ⁺ ratio	↓↔	↑↔	↓
Generation of cytotoxic T cells	↓	↑	↓
Plasma IL-2	↓	↑	↑, ↔
Plasma IFN-γ	↓	↑	?
Plasma IL-4	↔, ↓, ↑	?	↔
<i>In vitro</i> IL-2 secretion	↓, ↔	↑, ↔	↓
<i>In vitro</i> IFN-γ secretion	↓	?	↓, ↔
<i>In vitro</i> IL-4 secretion or mRNA	↑ (mRNA)	?	↑, ↔
Delayed-type hypersensitivity	↓	↑	↓, ↔
Antibody-dependent cytotoxicity	↓	↔	?
Splenic T-cell cytotoxicity	↓ (mice)	↑	?
Hydrolysis of PIP ₂ in T cells	↓	↑	?
Total protein kinase C activity in T cells	↓	↑	?
Protein kinase C translocation after T cell activation	↓	↑	Expected ↑
Protein kinase C mRNA expression in T cells	↓	↑	Expected ↑
NK cell activity	↓	↑	↓

↑ = increased; ↓ = decreased; ↔ = no change; ? = information not available.

Other cell-mediated immune responses impaired by iron deficiency and/or chelation include antibody-dependent cytotoxicity, spleen T cell and peritoneal cavity cell cytotoxicity against tumor cells, hydrolysis of cell membrane phospholipids, and protein kinase C activation and mRNA expression (Kuvibidila *et al.*, 1983b, 1998, 1999, Alcantara *et al.*, 1991; Oppenheimer, 2001). Iron therapy for one to three months corrects the affected immune responses.

7.2.2 The effects of iron deficiency on the secretion of pro- and anti-inflammatory cytokines

Iron deficiency appears to have a generalized negative effect on both pro- and antiinflammatory cytokines. In humans and laboratory animals, iron deficiency decreases levels in plasma and/or supernatant of activated lymphocytes of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, and IFN-γ (Galan *et al.*, 1992; Kuvibidila *et al.*, 1992, 2004, 2012; Omara and Blakley, 1994, Ekiz *et al.*, 2005; Nyakeriga *et al.*, 2005; Mbugi *et al.*, 2010; Suega and Bakta, 2010) (Table 7.3). Reduced secretion of IL-12 may explain the low levels of IFN-γ observed in the

Table 7.3 Effects of iron status on inflammatory cytokines and related factors

	Effect of iron deficiency	Effect of iron repletion	Effect of iron overload
<i>In vitro</i> IL-1 secretion	↓ (rats), ↔ (humans)	?	↓
<i>In vitro</i> TNF- α secretion	↓	?	↓, ↔
<i>In vitro</i> IL-10 secretion	↔, ↓	?	↑
Plasma IL-4	↔, ↓, ↑	?	↔
Plasma IL-5	↓ (malaria)	?	?
Plasma IL-6	↓	?	?
<i>In vitro</i> IL-8 secretion	↑ with iron chelation (HT29 cells)	?	?
Plasma IL-10	↓	?	↔
Plasma IL-12	↓	?	↔
Plasma IL-13	↓	?	?
Plasma TNF- α	?	?	↔
CD80 expression on resting and activated cells	↑	?	↔
CD86 expression on resting and activated cells	↑	?	↔
p38 MAPK in THP-1 macrophage cell line	↑ with iron chelation	?	?
NF κ B activation in lymphocytes	↓ (Lin <i>et al.</i> , 1997)	↑	↑
NF κ B activation in THP-1 macrophage cell line	↑ with iron chelation	?	?
PGE ₂ production by neutrophils	↑ with iron chelation	?	?
iNOS expression in HT29 cells	↑	↓	↔

iNOS = inducible nitric oxide synthase; PGE₂ = prostaglandin E₂; p38MAPK = mitogen activated kinase; ↑ = increased; ↓ = decreased; ↔ = no change; ? = information not available.

plasma and supernatant of activated mononuclear cells (Kuvibidila *et al.*, 2004, 2011). Iron repletion for one to three months corrects the reduced plasma IFN- γ concentrations and other cytokines (Suega and Bakta, 2010).

Occasionally, there are conflicting results regarding the effect of iron deficiency on cytokine mRNAs as compared to the proteins; examples of the cytokines are IL-1 β , IL-2, IL-4, IL-10, IFN- γ , and TNF- α (Bergman *et al.*, 2004; Nyakeriga *et al.*, 2005). Conflicting results may be due to mRNA stability and rate of translation. The effect of iron deficiency on cytokines is sometimes tissue- or cell type-specific. Contrary to what one would expect based on cytokine levels of plasma or supernatant of cultured cells, iron chelation by deferrioxamine increases NF κ B activation in THP1 (human monocytic) cells (Choi *et al.*, 2004), but decreases it in lymphocytes (Vallabhapurapu and Karim, 2009). Iron chelation has also been shown to increase IL-8 secretion by the HT29 intestinal cell line, which suggests that iron deficiency may increase inflammation in the gastrointestinal tract (Choi *et al.*, 2004). Iron chelation also increases the production of prostaglandin E₂ (PGE₂), an effect that may inhibit the secretion of pro-inflammatory cytokines by Th1 cells and macrophages (Fan *et al.*, 2010).

7.2.3 The effects of iron deficiency on humoral immunity

In contrast to T cells, resting B cells express low levels of transferrin receptor which implies that they continuously take up small quantities of iron from the environment (Neckers *et al.*, 1984). Upon activation, up to 80% of B cells express transferrin receptor, and hence exhibit iron uptake. This implies that iron deprivation may also affect certain B-cell functions. However, in general, indicators of humoral immunity are not affected by iron deficiency. The percentage of B cells and the concentrations of immunoglobulins (Ig) are either unchanged, slightly decreased, or slightly increased in iron deficiency, and antibody production in response to tetanus toxoid immunization is also normal (reviewed by Oppenheimer, 2001, Svoboda *et al.*, 2007; Sadeghian *et al.*, 2010) (Table 7.4). Considering that PGE₂ promotes TH2 cell differentiation which regulates antibody production, the lack or minimal effect of iron deficiency on humoral immunity may be the results of increased PGE₂ production. Additionally, increased PGE₂ which inhibits Th1 response, may also explain the impaired cell-mediated immunity often associated with iron deficiency.

The lack of an effect of iron deficiency on humoral immunity may also be related to other factors, such as the methods used to assess B-cell function, for example measuring total immunoglobulin (Ig) concentrations instead of Ig subclasses (Ekiz *et al.*, 2005). It is important to note that normal or elevated plasma Ig concentrations do not directly imply lack of defect in B-cell function, but rather could reflect previous and continuous exposure to pathogens. In fact, measurement of specific antibodies following vaccination, the quality of antibodies (antibody–antigen affinity), specific antibodies against T cell-dependent and T cell-independent antigens would be better tests for assessing humoral immunity than the measurement of total Ig. It is anticipated that production of antibodies against T cell-dependent antigens would be affected more than that of T cell-independent antigens. There is a paucity of data on the effects of iron deficiency on

Table 7.4 Effects of iron status on aspects of humoral immunity

	Effect of iron deficiency	Effect of iron repletion	Effect of iron overload
% B cells in blood	↓, ↑, ↔	↓	↑, ↔
Total B cell number in blood	↓, ↑, ↔	↔	↑, ↔
Total serum immunoglobulins	↑, ↔	↓	↓, ↔
Intestinal sIgA in rodents	↔	No change expected	?
Serum IgG1; IgG2; IgG3	↔	No change expected	?
Serum IgG4	↓	Expected to increase	?
Serum IgE	↓ (malaria)	Expected to increase	?
Ig secretion by B cells	↓ (rodents)	Expected to increase	?
Serum IgM	↓	Expected to increase	?
B-cell proliferation	↓	↑	?
Antibody response to tetanus toxoid and influenza vaccine in rodents	↓	Expected to increase	↑

↑ = increased; ↓ = decreased; ↔ = no change; ? = information not available.

antibody quality. One study has reported reduced plasma levels of IgG4 (though normal values of all other Ig subtypes) in iron-deficient individuals (Ekiz *et al.*, 2005). Considering that IgG4 is crucial in the clearance of *Cryptococcus neoformans*, the decreased plasma IgG4 levels may increase the risk of morbidity and mortality in iron-deficient immune-compromised individuals (Beenhouwer *et al.*, 2007).

In contrast to humans, iron deficiency in laboratory animals decreases the percentage of B cells in the spleen (reviewed by Oppenheimer, 2001), B-cell proliferation in response to bacterial lipopolysaccharide (Kuvibidila *et al.*, 1983a), antibody responses to influenza vaccine (Dhur *et al.*, 1990), the number of plaque-forming cells (Kuvibidila *et al.*, 1982), Ig levels, and the number of intestinal cells containing IgM and (secretory) sIgA (Perkkiö *et al.*, 1987). Reduced number of IgM and sIgA containing cells may affect intestinal mucosal immunity.

7.2.4 The effects of iron deficiency on macrophage functions

Quiescent monocytes from healthy individuals do not express transferrin receptor (Testa *et al.*, 1991). However, more than 90% of macrophages, especially those from the lungs, express transferrin receptor (Andreesen *et al.*, 1984; Testa *et al.*, 1991). Macrophages differ from other immune and non-immune cells because they upregulate the expression of surface transferrin receptor when cultured in an iron-rich medium. This makes sense because macrophages are involved in iron storage and require iron for cytotoxic activity against various pathogens.

Iron deficiency and iron chelation in human macrophages inhibit pathogen phagocytosis, pathogen cytotoxicity, tumoricidal activity, and the secretion of macrophage migration inhibitory factor and TNF- α , but not IL-1 (Swarup-Mitra and Sinha, 1984; O'Brien-Ladner *et al.*, 1998) (Table 7.5). In laboratory mice, iron deficiency also decreases the cytotoxic activity of mononuclear cells from peritoneal cavity against tumor cells, IL-1 secretion, and the clearance of foreign particles (Kuvibidila *et al.*, 1983b; Hallquist *et al.*, 1992). Reduced cytotoxicity is very likely due to reduced production of reactive oxygen species.

7.2.5 The effects of iron deficiency on neutrophil functions

Contradictory information exists in the literature about the expression of transferrin receptors on non-activated neutrophils, with some studies showing no detection of the receptors, while others showing the opposite (Maneva and Taleva, 2009; Table 5). While neutrophil phagocytosis is either normal or decreased during iron deficiency, intracellular bacteria killing is always significantly impaired (Walter *et al.*, 1986; Murakawa *et al.*, 1987; Bergman *et al.*, 2005). In parallel with reduced bactericidal activity, the activity of myeloperoxidase, an iron-dependent enzyme involved in neutrophil bactericidal activity, is impaired. The impaired neutrophil functions are corrected within a few weeks of iron repletion.

7.2.6 The effects of natural killer (NK) cell activity

Resting NK cells do not express surface transferrin receptors, and they probably take up very little iron from the medium or they do so through an unknown

Table 7.5 Effects of iron status on aspects of innate immunity

	Effect of iron deficiency	Effect of iron repletion	Effect of iron overload
Macrophage/monocyte phagocytosis	↓	?	↓, ↔ (Walker and Walker, 2000)
Macrophage/monocyte cytotoxicity	↓	?	↓, ↔, ↓
Peritoneal macrophage tumoricidal activity	↓	?	?
Neutrophil phagocytosis	↓, ↔	↔	↓
Neutrophil bactericidal activity	↓	↑	↓
Neutrophil myeloperoxidase activity	↓	↑	?
Neutrophil chemotaxis	↓	↑	?
Natural killer cell activity	↓	↑	↓
<i>In vivo</i> macrophage clearance of particles	↓	↑	?
Nitroblue reduction by macrophages	↓, ↔	?	↓
Zymosan opsonization	?	?	↓
Nitric oxide production	↓	↑	↓ (neutrophils) ↔ (macrophages)
Serum complement C3	↓	↑	?
Hemolytic activity, CH50	↓, ↔	↑	?

↑ = increased; ↓ = decreased; ↔ = no change; ? = information not available. CH50, 50% hemolytic complement.

transferrin receptor-independent pathway (Kemp, 1993). However, upon activation, they express the transferrin receptor on their surface. The limited available data suggest that iron deficiency decreases NK cell activity (Spear and Sherman, 1992).

7.3 The effects of iron overload on immunity

7.3.1 Iron overload and lymphocyte functions

As indicated in Tables 7.2–7.5, iron overload may also induce immune dysfunction. Iron overload may reduce total white blood cell counts, percentage of CD4⁺ and/or CD8⁺ lymphocytes, CD4⁺/CD8⁺ ratio, *in vitro* IL-2 secretion, CD8⁺ cytotoxicity, and delayed type hypersensitivity (Kaplan *et al.*, 1984; Wu *et al.*, 1990; Tsouchnikas *et al.*, 2007). In patients with hereditary hemochromatosis, but non-genetic iron overload (as observed in the Bantu of South Africa), while the number of CD8⁺/CD28⁺ is reduced, that of CD8⁺/CD28⁻ is increased and that of CD4⁺/CD28⁺ is unaffected (Porto and De Sousa, 2007). Reduced subpopulation of CD8⁺/28⁺ (suppressor) cells may predispose to infection. While iron overload has no effect on the secretion of IFN-γ by CD4⁺ lymphocytes, it increases that of IL-4 in the same cells (Mencacci *et al.*, 1997; Fabio *et al.*, 2002). In CD8⁺ cells, iron overload increases the secretion of IFN-γ, IL-4 and IL-10 (Fabio *et al.*, 2002). The increase in IL-4 and IL-10 secretion is necessary to dampen inflammation usually

induced by free radicals. The discrepancy between various T-cell subsets may be related to intracellular iron levels of CD4⁺ and CD8⁺ lymphocytes or the threshold of iron levels required to alter the immune response.

7.3.2 Iron overload and macrophage functions

Iron is required for the generation of hydroxyl radicals, which are more potent antimicrobial agents than hydrogen peroxide and superoxide anion which are produced during the oxidative burst. The effect of iron overload on macrophage function is controversial. Although phagocytosis (of yeast) is unaltered, the secretion of nitric oxide, IL-12, IL-1 β and IL-6 is either decreased, increased, or unaffected (Saleppico *et al.*, 1996; O'Brien-Ladner *et al.*, 1998; Serafin-Lopez *et al.*, 2004; Wang *et al.*, 2008; Table 7.5). However, when iron is added to the culture medium, macrophage killing capacity of certain types of bacteria (*Bruccella abortus*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*), is increased (Jiang and Baldwin, 1993; Byrd, 1997). The conflicting results may be explained by four factors: (i) the source of macrophages (human subjects with hemochromatosis, blood transfused patients, laboratory animals fed diets with very high iron content); (ii) the tissue origin of the cells (blood, peritoneal cavity, lung, cell lines incubated in medium with high iron content); (iii) the degree of iron loading; and (iv) the nature of the pathogen investigated. There is a paucity of data on the effects of iron (deficiency or overload) on dendritic cell function.

7.3.3 Iron overload and neutrophils

Iron overload due to multiple blood transfusions in patients with β -thalassemia (Dwyer *et al.*, 1987) and those with renal disease (Waterlot *et al.*, 1985) reduces phagocytosis of yeast, *S. aureus*, and *Escherichia coli*, nitroblue tetrazolium reduction, zymosan opsonization, myeloperoxidase activity, and bactericidal capacity (Cantineaux *et al.*, 1987, 1999). In laboratory mice, iron overload inhibits the secretion of NO and IL-12 by neutrophils, an effect that may promote infection (Mencacci *et al.*, 1997). Iron chelation restores the impaired function.

7.3.4 Iron overload and NK cell functions

Patients with hereditary hemochromatosis have reduced mean percentage and absolute numbers of NK⁺ cells, together with NK cell cytotoxicity (Fabio *et al.*, 2002). Iron overload resulting from blood transfusion in patients with β -thalassemia or sickle cell disease depresses NK cell activity (Kaplan *et al.*, 1984; Akbar *et al.*, 1986; Chapman *et al.*, 1988;). However, while *in vitro* iron chelation by desferrioxamine improves NK cell activity, administration of the same compound to patients does not correct it. The lack of improvement in NK cell activity may be due to either poor uptake of the chelator and thus failure to significantly reduce iron cellular levels, or the presence of confounding factors unrelated to iron levels. The

decreased NK cell number and/or activity may increase the risk of viral infection and perhaps impair tumor killing.

7.4 Mechanisms of impaired immunity in iron deficiency

The mechanisms by which iron deficiency impairs cell-mediated and non-specific immunity are multifactorial and continue to be elucidated (Table 7.6). They include, but are not limited to, reduced activity of iron-dependent enzymes (specifically ribonucleotide reductase, myeloperoxidase activity), reduced cytokine gene expression and/or cytokine secretion, reduced number of immunocompetent T cells and altered signal transduction. Related to reduced number of immunocompetent T lymphocytes, it was recently observed that the percentage of CD1⁺ cells (a marker of immature thymocytes) in peripheral blood lymphocytes was nearly six-fold higher in iron-deficient Bolivian children than in age-matched controls and it decreased after iron supplementation (Sejas *et al.*, 2008).

Several steps of signal-transduction pathways that are regulated by iron have been described and perhaps several other pathways remain to be described. One of the earliest events associated with lymphocyte activation occurring within seconds of antigen/mitogen-binding to specific cell receptors is the hydrolysis of cell membrane phosphatidyl-inositol 4,5 bisphosphate by phospholipase C. The reaction leads to the production of diacylglycerol (DAG) and inositol 1,3,5 triphosphate (IP₃). DAG is involved in protein kinase C (PKC) activation and IP₃ in the release of sequestered calcium from endoplasmic reticulum. DAG and IP₃ regulate T lymphocyte proliferation, cytokine gene expression, and subsequently lymphocyte functions. We now know that iron deficiency impairs PIP₂ hydrolysis (Kuvibidila *et al.*, 1998). It is to be added that phospholipase C, one of the enzymes or family of enzymes involved in PIP₂ hydrolysis, is a zinc-dependent enzyme, not an iron-dependent enzyme, which implies that the role of iron on this enzyme may be indirect, perhaps at the level of gene expression. This information, however, is not available at the present time.

PKC total activity and its translocation to the plasma membrane in murine spleen lymphocytes and human T-cell lines are also impaired by iron deficiency and/or iron chelation (Alcantara *et al.*, 1991; Kuvibidila *et al.*, 1991, 1999). Impaired PKC translocation is not surprising considering impaired PIP₂ hydrolysis or reduced DAG production which is required for the translocation. As for phospholipase C, PKC is not an iron-dependent enzyme; therefore the role of iron on this enzyme is indirect. It turns out that iron regulates PKC gene expression and, in fact, iron chelation reduces PKC mRNA expression (Alcantara *et al.*, 1991).

During progression of lymphocytes through the cell cycle, the expression of cell cycle regulator proteins – cyclins A, B, and cyclin-dependent kinases – is usually increased (Lucas *et al.*, 1995). Activated and/or proliferating lymphocytes normally express cyclins A, B, and E, and related kinases. Iron chelation in peripheral blood lymphocytes obtained from healthy volunteers suppresses the expression of cyclin A and cyclin-dependent kinases 2 and 4 and arrests cells in the G0/G1 phase

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Table 7.6 Possible mechanisms of impaired cell-mediated immunity during iron deficiency and/or iron overload

Effect	Impact
Decreased proportion and absolute number of immunocompetent T cells (↑ CD1 ⁺ cells)	Fewer T-cells to respond to challenge (antigen) (Attia <i>et al.</i> , 2009)
Decreased activity of ribonucleotide reductase	Decreased proliferation of T cells
Decreased activity of other iron-dependent enzymes, including myeloperoxidases	Impaired bactericidal activity of neutrophils
Altered composition of cell-membrane phospholipids	Altered responsiveness of (T) cells
Decreased hydrolysis of cell-membrane phospholipids (PIP ₂ in T cells)	Decreased responsiveness of T cells (Kuvibidila, <i>et al.</i> , 1998)
Decreased protein kinase C translocation to cell membrane (activation)	Decreased responsiveness of T cells (Kuvibidila <i>et al.</i> , 1999)
Decreased activity of other protein kinases (i.e. calmodulin) that phosphorylate various factors that regulate immune cell proliferation	Decreased responsiveness of T cells
Impaired release of sequestered (endoplasmic reticulum) calcium and/or transfer from extracellular pool to the cytoplasm	Decreased responsiveness of T cells
Altered expression of T-cell receptor (TCR/CD3)	Decreased responsiveness of T cells (Kuvibidila and Porretta, 2003)
Decreased expression of CD28?	Decreased responsiveness of T cells (Kuvibidila and Porretta, 2003)
Altered expression of CD80 and CD86 on antigen presenting cells: in mice, the expression of these molecules is increased possibly to compensate for the decreased CD28 expression	Increased expression by iron chelation/deficiency may be a compensatory mechanism to upregulate the co-stimulatory pathway and overall T lymphocyte response to stimuli (Kuvibidila and Porretta, 2002)
Decreased antigen presenting cell function by B cells, macrophages, monocytes and/or dendritic cells	Decreased activation of T cells → decreased responsiveness of T cells and NK cells
Decreased NFκB activation	Decreased production of inflammatory cytokines
Increased iNOS activity	Increased secretion of nitric oxide → increased microbial killing
Altered COX-2 activity	Altered PGE ₂ , impaired Th1 response and macrophage functions; increased B-cell response
Altered cyclins and cyclin-dependent kinases	Decreased T- and B-cell proliferation (Lucas <i>et al.</i> , 1995)

(Lucas *et al.*, 1995). Since cyclin A triggers the entry of cells into the S phase, reduced expression of cyclin A and cyclin A-dependent kinase by iron chelation may explain reduced proliferation of iron-deficient lymphocytes.

The other system that explains impaired immunity during iron deficiency is the inverse response of NF κ B and inducible nitric oxide synthase (iNOS). In normal and elevated iron intracellular levels, NF κ B expression is increased via high production of reactive oxygen species which, in turn, leads to increased cytokine gene expression and cytokine secretion. During iron deficiency, the production of reactive oxygen species is reduced, and so is NF κ B activation and cytokine secretion. The iNOS, on the other hand, is upregulated by iron chelation and downregulated by normal or elevated iron, probably to counteract the effect of NF κ B activity (Cherayil BJ, 2010).

7.5 Iron status and infection

The role of iron in the proliferation of eukaryotic cells and in many vital biochemical and cellular functions including energy metabolism, detoxification of toxins and drugs, and the metabolism of amino acids and vitamins has been well established. Some of these functions directly or indirectly modulate immune function and defense against infection. As an example, iron is required for the metabolism of vitamin D, specifically the activation of vitamin D₃ to 25(OH)-vitamin D in the liver and to 1 α ,25(OH)₂ vitamin D in the kidney and macrophages (Araya *et al.*, 2003). The 1 α ,25(OH)₂ vitamin D (secreted by macrophages) is an excellent inducer of defensins, which are very potent antipathogens (Wang *et al.*, 2004; White, 2008; Youssef *et al.*, 2011). In theory, iron deficiency severe enough to impair 1 α ,25(OH)₂ vitamin D synthesis and perhaps defensins production by macrophages may therefore increase the risk of infection independent of the impact on the functions of other immune cells.

The role of iron in cell division and cellular functions is well established. Almost all living cells, including bacteria, fungi, protozoa, and mammalian cells from various tissues, including lymphoid organs require iron for DNA synthesis and many other cellular functions. Iron is an integral part of ribonucleotide reductase (RNR), the key enzyme in the biosynthesis of deoxyribonucleotides, the backbone of DNA. In addition to its role in RNR, iron is also a co-factor for myeloperoxidase – a key enzyme for neutrophil bactericidal activity. It is also a co-factor in proteins or enzymes involved in the production of reactive oxygen species, and of catalase that has a role in antioxidant defense. Micro-organisms need iron in the range of 22–220 μ g/l (Payne and Finkelstein, 1978). Although low iron levels will diminish the growth of micro-organisms (Patruta and Horl, 1999), the concentration in body fluids and cell compartments is more than enough to sustain optimal growth of micro-organisms. However, this iron is tightly bound to various proteins (hemoglobin, ferritin, transferrin, lactoferrin, myoglobin, and various enzymes), and therefore in general it is unavailable to pathogens. On the host's side, too little iron may impair immune functions, especially those that

require clonal expansion. Too much iron induces oxidative stress which is harmful to host cell proteins, membrane fatty acids and nucleic acids. As explained in an earlier section, iron overload also impairs various aspects of immune function. Thus, there must be a suitable concentration of iron – enough to support the immune function, while not promoting the growth of infectious agents.

The importance of this subtle balance probably explains the variety of observations that have been made with regard to the influence of iron status on susceptibility to infections. Some observations demonstrate that iron (supplementation) promotes infection, suggesting that iron deficiency is beneficial, as it may protect from illnesses (Oppenheimer, 2001). Other observations show that iron supplementation or adequate iron status (defined by various biochemical indices) may protect from infection, which implies that iron deficiency is deleterious and may promote infection. The second view may go along with the impaired innate and cell-mediated immunity often seen in subjects with iron deficiency. Yet other observations indicate that iron status alone is probably insufficient to determine susceptibility to infection (Snow *et al.*, 1991; Heresi *et al.*, 1995; Menendez *et al.*, 1997). In fact, there is no evidence that the use of iron-fortified milk during the first year of life in children alters the rate of diarrheal diseases and respiratory infections (Heresi *et al.*, 1995).

7.5.1 Evidence that iron may promote infection

A consideration of the effects of iron status or supplementation on infection has been the subject of several reviews (Oppenheimer, 2001; Schaible and Kaufmann, 2004; Markel *et al.*, 2007; Kumar *et al.*, 2010). Several published studies suggest that administration of iron to neonates or malnourished individuals increases morbidity and mortality due to bacterial and/or malaria infections (reviewed by Smith *et al.*, 1989; Oppenheimer, 2001; Wander *et al.*, 2009). In support of the idea that iron promotes infections is the observation that during infection, the host responds by decreasing serum iron concentration and shifting to storage in the reticuloendothelial cells; it may be that this is an attempt by the host to deprive invading pathogens of iron. One of the characteristics of infection is the rapid increase in the biosynthesis of inflammatory cytokines, including IL-1, IL-6, TNF- α , and IFN- γ , various acute-phase proteins, such as C-reactive protein, α 1-acid glycoprotein, and stress hormones such as cortisol, etc. Unfortunately, the acute-phase response and increased body temperature are also associated with reduced absorption or plasma levels of several other micronutrients (zinc, vitamin A, folate, vitamin B12), and these nutrients all modulate immunity.

In contrast to bacteria that can invade many different cell types with limited intracellular iron, and in plasma or other body fluid, malaria parasites are different because they are totally dependent upon young red blood cells of the host to complete the lifecycle. Needless to say, the red blood cells are extremely rich in heme iron. The favorable milieu may explain the observation that malaria is more common in iron-replete than in iron-deficient individuals (Oppenheimer *et al.*,

1986; Smith *et al.*, 1989) and that the levels of malaria infection and the severity are increased by iron supplementation (Murray *et al.*, 1978).

7.5.2 Evidence that iron may protect from infection and iron deficiency may promote infection

Several published studies suggest that that prevention and treatment of iron deficiency by medicinal iron and food fortification reduces the rate of respiratory and non-respiratory infections (reviewed by Oppenheimer, 2001). The mechanism of improvement may rely in the role of iron on cell-mediated immunity, activation of NFκB, and production of free radicals and pro-inflammatory cytokines that have antimicrobial properties.

7.6 Conclusion

In spite of the numerous human and laboratory animal studies showing that innate and cell-mediated immune responses are in general impaired in iron deficiency, the relationship between iron deficiency and infection is far less clear. Unfortunately, the issue of susceptibility to infection is very complex and depends not only on iron status, but also on many host, pathogen, and environmental factors. Some of these factors include exposure to micro-organisms, the type of micro-organisms (intracellular versus extracellular bacteria, viruses, fungi, parasites), the presence of other nutritional deficiencies, the study population (neonates, young children, women, men, the elderly), the severity and duration of iron deficiency, the form, dose, and duration of iron therapy, and pre-existing conditions (primary and secondary immune deficiencies). It must be recognized that an association does not mean a cause and direct effect. However, it is clear that, due to the role of iron on immune cell proliferation and production of antimicrobial compounds, both iron deficiency and iron overload will affect susceptibility to certain types of infection. Iron overload may increase the risk of infection due to intracellular and extracellular pathogens. Oral and intramuscular administration of therapeutic doses of iron to immunocompromised (malnourished) individuals is associated with increased risk of morbidity due to malaria and other infectious diseases and therefore should be avoided. Considering the many deleterious effects of iron deficiency independent of immunity, public health authorities can still use either food fortification or targeted iron supplements of high-risk groups to prevent this nutritional deficiency.

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8

Vitamin A and the immune system

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Abstract:: Vitamin A is required in the diet of all vertebrates, and is metabolized intracellularly to retinoic acid (RA), which is well known as a regulator of cell proliferation and differentiation. RA activates nuclear retinoid receptors and regulates a wide variety of genes. This chapter first discusses vitamin A nutrition and metabolism, and then how inflammation affects the metabolism of vitamin A compounds. Clinically, RA and several analogs are used in the treatment of skin disorders. Vitamin A deficiency is a risk factor for infectious disease. This chapter discusses the role of vitamin A in the immune system, especially in the regulation of T cell differentiation and in innate immunity.

Key words: antigen-presenting cells, dendritic cells, inflammation, retinol, retinol-binding protein, retinoic acid, retinoid homeostasis, T cells, T cell homing, transcription factors.

8.1 Introduction

Vitamin A (retinol) is an essential micronutrient for all vertebrates. Retinol is metabolized to an active hormone-like molecule, retinoic acid (RA), a potent regulator of cell differentiation with effects in all organ systems. Due to its ability to promote cell differentiation, RA has long been of interest in cancer prevention and therapy (Altucci and Gronemeyer, 2001; Fields *et al.*, 2007; Ross, 2010). This chapter on vitamin A, inflammation and immunity focuses first on the sources of vitamin A in the diet, the ways in which vitamin A is metabolized to generate RA, and how the metabolism of vitamin A is itself affected by inflammation. The chapter then discusses the role of vitamin A and RA in the skin, where RA and retinoid analogs have been used to treat inflammatory conditions, such as acne. In the final section of this chapter, we turn attention to vitamin A and immunity,

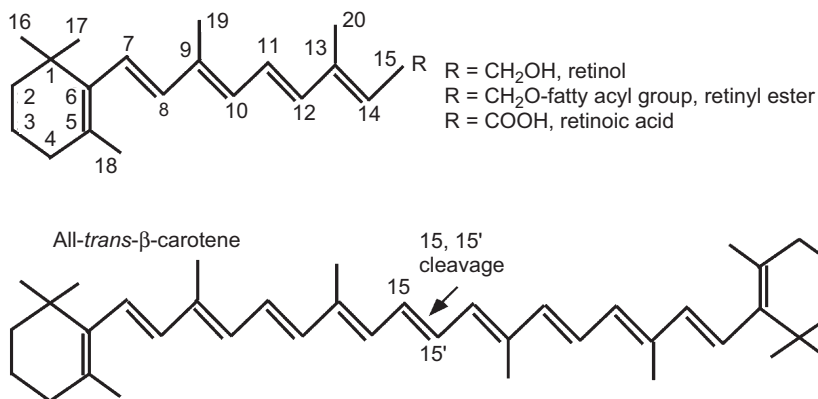


Fig. 8.1 Vitamin A (retinol) and its major derivatives, and β-carotene. Both are shown in their all-*trans* configurations. Many minor retinoids and isomers have also been detected in plasma and tissues.

focusing on recent research that has helped define how vitamin A status, and RA specifically, regulates immune responses, especially the differentiation of T lymphocytes and the processes they regulate, which are necessary for intestinal mucosal immunity, and innate immune responses necessary for virus clearance after viral infection.

8.2 Sources, forms and dietary recommendations for vitamin A

8.2.1 Nutritional forms

Most human diets contain vitamin A in two forms: retinol (Fig. 8.1a), also referred to as preformed vitamin A, and provitamin A carotenoids. Retinol is present mostly as retinyl esters in relatively high concentrations in organ meats and in lower concentrations in dairy products (US Offices of Dietary Supplements, 2012). Provitamin A comprises three principal carotenoids: β-carotene (Fig. 8.1b), α-carotene, and β-cryptoxanthin, which are present in varying amounts in leafy green and yellow vegetables and fruits such as mango, papaya and oranges. In western mixed diets, about two-thirds of total dietary vitamin A is consumed as preformed vitamin A, while in developing countries where the consumption of animal foods is generally much lower, carotenoids provide most of the total vitamin A, and total intakes of vitamin A are also often low.

8.2.2 Recommended intakes

The Recommended Dietary Allowance established in the USA by the Institute of Medicine (IOM), is 700 and 900 μg retinol equivalents (RAE) per day for adult men and women, respectively, and proportionately lower for children (Institute of

Medicine, 2001). Other organizations have set slightly lower recommended nutrient intakes, for example the WHO recommendation is 500–600 µg equivalents of retinol per day for adults (WHO, 1998). The IOM has also defined a tolerable upper intake level (UL), set at 3000 µg of retinol per day for adults. Intakes of preformed retinol of >10 000 µg/d have been associated with an increased risk of birth defects (reviewed in Institute of Medicine, 2001), and thus the UL was set low enough to minimize risk of teratogenicity in women of child-bearing age, as well as to minimize the risk of vitamin A-induced liver disease in all age–sex groups. It should be noted that β-carotene, in contrast to retinol, poses little risk of adverse effects and therefore is considered to be a safe, although less efficient, source of vitamin A (Grune *et al.*, 2010).

Vitamin A deficiency is still a public health problem in many parts of the developing world, including India, Southeast Asia, Africa and Central America. Improving vitamin A status in such regions is one strategy of the Millennium Development Goals of UNICEF to reduce child mortality (UNICEF, 2013). The WHO actively encourages programs to deliver supplemental vitamin A to young children in at-risk populations (IVACG, 1993). Vitamin A supplementation programs generally provide vitamin A once every four to six months in capsule form, where each dose contains 30–60 mg of retinol, as retinyl palmitate, depending on the child's age (WHO, 2011). This strategy is effective because, as discussed in Section 8.3, vitamin A is readily absorbed and stored in the liver, from which it can be released over time to provide sufficient retinol for tissues including the eyes where retinol is converted to 11-*cis*-retinal and used for synthesis of rhodopsin, as required for vision, and many other tissues for proper cell differentiation, tissue maintenance and repair.

8.2.3 Pharmacological forms

The acidic form of vitamin A, RA, and numerous structurally related compounds (generally termed retinoids) have been produced synthetically, and some have been approved as drugs. Several retinoids have proved to be very effective therapeutically in certain diseases; for example, all-*trans*-RA is effective in the treatment of acute promyelocytic leukemia through its ability to induce promyelocyte differentiation, while 13-*cis*-RA and various retinoid analogs are effective in the treatment of diseases such as of the skin (see further in Section 8.4).

8.3 Major aspects of normal vitamin A metabolism

8.3.1 Uptake and distribution of newly absorbed and stored vitamin A

As illustrated in Fig. 8.2, the metabolism of both preformed vitamin A and provitamin A carotenoids begins in the intestine, where the majority of β-carotene is first converted into retinal by the cleavage enzyme β-carotene monooxygenase-1 (BCO-1) (von Lintig *et al.*, 2005, Lietz *et al.*, 2010), followed by reduction to

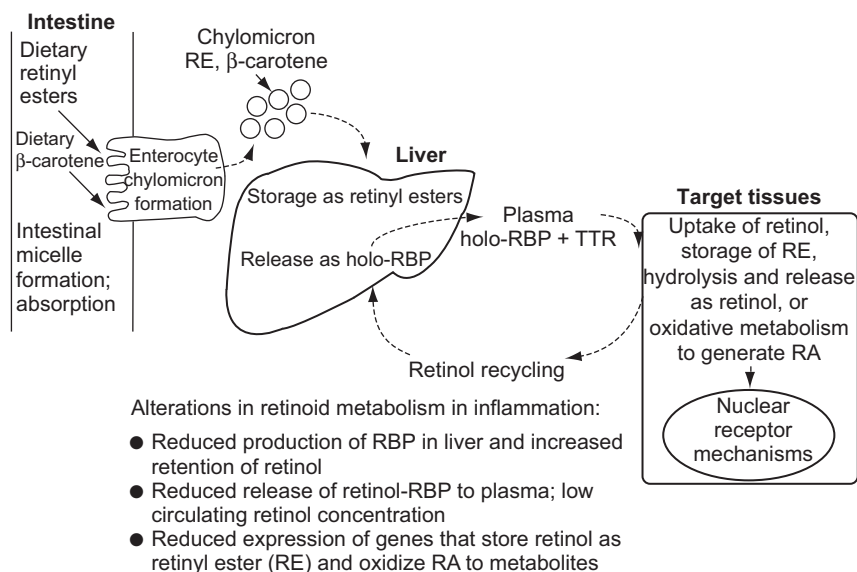


Fig. 8.2 Schematic view of major processes in vitamin A metabolism, and summary of effects of inflammation and infection on the transport of retinol.

retinol. Retinol from either preformed vitamin A or β -carotene cleavage is then esterified and packaged with triglycerides and other lipids into chylomicrons. In humans and some animals, a portion of β -carotene, possibly ~20%, is absorbed intact, without cleavage, and packaged into chylomicrons along with retinyl esters. Therefore the amounts and relative proportions of retinyl esters and β -carotene carried in chylomicrons depend on both intake and the efficiency of β -carotene metabolism, which varies considerably among individuals (Tang *et al.*, 2003; Lietz *et al.*, 2010; Grune *et al.*, 2010). In plasma, chylomicron triglycerides are rapidly hydrolyzed by lipoprotein lipase, while the majority of esterified retinol remains within the chylomicron remnant and is taken up by the liver during remnant uptake, with lesser amounts going to adipose, lung and other tissues. Within the liver, the newly absorbed vitamin A can either be stored, mainly in hepatic stellate cells, or rapidly secreted back into plasma as retinol bound to retinol-binding protein (holo-RBP), which associates with the co-transport protein transthyretin (TTR). The process of vitamin A storage predominates when vitamin A status is adequate, whereas, in contrast, retinol is rapidly secreted into plasma as holo-RBP when vitamin A is administered to vitamin A-deficient animals (Muto *et al.*, 1972), and similarly in humans. Overall, an individual's vitamin A status is a strong determinant of the fate of newly absorbed vitamin A, especially at the level of hepatic metabolism.

Normally, plasma retinol levels are tightly controlled by a network of homeostatic mechanisms that are very effective as long as the liver has a sufficient supply of stored vitamin A to maintain its normal rate of secretion. Thus, the person-to-person and day-to-day variation in plasma retinol levels is relatively small. Some

tissues such as the retina (Kawaguchi *et al.*, 2007) and lungs (Wu and Ross, 2010) contain a receptor for RBP, known as Stra6, which facilitates retinol uptake from plasma. The body's vitamin A pool tends to be well conserved, as demonstrated in kinetic studies which have indicated an extensive recycling of retinol between plasma, liver and extrahepatic tissues before retinol undergoes irreversible disposal via oxidative metabolism (Cifelli *et al.*, 2007, Ross and Zolfaghari, 2011).

8.3.2 Other forms of vitamin A that may contribute to tissue retinoids

Besides retinol, other vitamin A molecules in plasma include retinyl esters in serum lipoproteins and various oxidized forms of vitamin A (O'Byrne and Blaner, 2005). Retinyl esters are usually present at low concentration (<10% compared to plasma retinol of 1–3 $\mu\text{mol/L}$). The plasma level of retinyl ester increases shortly after a high intake of vitamin A and may remain high, even exceeding the concentration of unesterified retinol, during hypervitaminosis A (Ross, 2005). Lipoprotein-bound retinyl esters can be taken into cells by a variety of processes including during lipoprotein uptake by low- and high-density lipoprotein receptors and scavenger receptors (O'Byrne and Blaner, 2005).

Carboxylic acid metabolites of vitamin A present in plasma include all-*trans*-RA, 13-*cis*-RA, the 4-oxo-forms of these retinoids, and their glucuronide conjugates. Whereas RA is transported by albumin, the more polar compounds are progressively more soluble in aqueous media and may not require a carrier. Acidic retinoids are usually present in the low nanomolar range, or about 1–2% as compared to plasma retinol. However, because the turnover rate of RA is very rapid, with a plasma half-life of minutes to hours, circulating RA can potentially contribute significantly to tissue retinoid uptake. Experimentally, RA has been shown to satisfy the requirements of most tissues for vitamin A (Dowling and Wald, 1960). Many minor retinoids have been detected in plasma and tissues at low levels, but their biological significance is uncertain.

8.3.3 Enzymatic processing and nuclear functions

Many tissues and cell types contain enzymes capable of oxidizing retinol to RA and more polar metabolites (Ross *et al.*, 2001, O'Byrne and Blaner, 2005). The net retinoid status of a given tissue is determined by a balance between retinoid uptake from plasma into cells, intracellular metabolism and elimination of retinoids through degradation or release back into plasma.

The intracellular metabolism of vitamin A is controlled in part by specific proteins which serve as chaperones (Noy, 2000), while the genomic actions of RA are mediated by nuclear retinoid receptors that function as transcription factors for retinoid-regulated genes (Bastien and Rochette-Egly, 2004; Blomhoff and Blomhoff, 2006). Proteins of the cellular retinol/retinoic acid-binding protein families (CRBP/CRABP) selectively bind all-*trans*-retinol and all-*trans*-RA and direct their cargo to specific enzymes that metabolize the bound retinoid, through esterification, oxidation and reduction reactions (Noy, 2000; Ross and Zolfaghari,

2004; O'Byrne and Blaner, 2005). The oxidation of retinol to retinal, followed by the irreversible oxidation of retinal produces all-*trans*-RA. In the nucleus, RA may bind to nuclear receptors of the RAR family (RAR- α , RAR- β , and RAR- γ), which in turn are associated with RXR proteins. The RAR–RXR complex binds to retinoic acid response elements (RAREs) in the promoter region or sometimes in other regions of retinoid-regulated genes (Bastien and Rochette-Egly, 2004; Blomhoff and Blomhoff, 2006). Although over 300 genes are physiologically regulated by RA, a direct transcriptional mechanism in which ligand activates an RAR/RXR bound to an RARE has been shown for relatively few of them, and some retinoid-responsive genes may be regulated indirectly (Balmer and Blomhoff, 2002). Non-genomic effects of RA have been proposed but are not firmly established.

Tissue retinoid levels are also controlled by oxidation, especially by cytochrome P450s of the CYP26 family, which form polar metabolites (Cifelli and Ross, 2007; Ross and Zolfaghari, 2011). Several genes that function in the metabolism of retinol, retinal and RA are regulated in part by RA in a feedback or feed-forward manner, forming a network of homeostatically regulated reactions that serve to tightly control RA levels under physiological conditions (Ross and Zolfaghari, 2011; Ross *et al.*, 2011b). This homeostatic system can, however, become overwhelmed when the intake of dietary vitamin A or the use of RA is excessive, with consequent development of retinoid toxicity.

8.4 Influence of inflammation on vitamin A transport and metabolism

Not only is vitamin A important for a well-regulated immune system and appropriate control of inflammation, as discussed later, but inflammation affects the transport and metabolism of retinol. Figure 8.2 denotes some of the changes that have been observed in retinoid homeostasis during inflammation.

8.4.1 Plasma retinol reduction

Plasma retinol concentration is reduced during infections, such as with measles; for example, the reduction of plasma retinol in Nigerian children with measles was even greater than in those with malnutrition, and some children experienced post-measles corneal ulceration, thought to be due to severe vitamin A deficiency (VAD) (Inua *et al.*, 1983). Infection-related reductions in plasma retinol can confound the interpretation of plasma retinol levels in various populations because the WHO defines subclinical VAD as a serum retinol < 0.7 $\mu\text{mol/L}$. Values in this range may result from infection or inflammatory conditions, even if retinol intake and tissue retinol storage are adequate: thus, the interpretation of low plasma retinol, when infection is known or suspected, may be problematic. Studies in animals with adequate vitamin A status have shown that administration of lipopolysaccharide (LPS), given to induce mild inflammation, results in a 40–50%

reduction in plasma retinol within 6–12 hours, with levels remaining low for approximately 48 hours before returning to normal (Rosales *et al.*, 1996). RBP mRNA was significantly reduced in the liver of LPS-treated animals, as was RBP protein (Rosales *et al.*, 1996). These results classify RBP as a negative acute-phase protein. In rats with marginal vitamin A storage and low plasma retinol, LPS-induced inflammation further reduced plasma retinol into a range that would be classified as deficient in humans (Rosales and Ross, 1998).

Other animal studies have shown disturbances in retinol kinetics during inflammation induced by either LPS or administration of recombinant human interleukin (IL)-6, the cytokine most responsible for the hepatic acute-phase response. For either treatment and especially after IL-6, which was delivered by sustained infusion for five days, the ^3H -retinol used to trace plasma retinol rapidly disappeared from plasma, consistent with reduced plasma retinol concentrations, apparently due to sequestration of retinol in tissues (Gieng *et al.*, 2007). The sequestration was reversible since, after the rats had recovered from inflammation, the plasma retinol concentration and plasma ^3H -retinol kinetics returned to control values. By using compartmental modeling it was possible to estimate that LPS induced a 79% reduction in hepatic mobilization of retinol by 15 hours while IL-6 caused an earlier reduction of 75% by 5.6 hours. The model also predicted a return to control values of plasma retinol by 10 days after inflammation (Gieng *et al.*, 2007). These data suggest that inflammation alters the plasma and tissue dynamics of vitamin A, without necessarily increasing retinol catabolism.

8.4.2 Studies in humans

Various studies in children and adults have also demonstrated that infection or inflammation affects retinol transport and metabolism. In children with malaria, retinol was inversely related to acute-phase protein concentrations (Rosales *et al.*, 2000). In children with pneumonia, serum C-reactive protein (CRP), an acute-phase protein and biomarker of inflammation, was inversely associated with retinol at both admission and discharge (Stephensen *et al.*, 2002a). Additionally, the Relative Dose Response test, an assessment tool used to indicate whether the liver has sufficient retinol for normal holo-RBP secretion, failed in children with CRP > 10 mg/L, even after they received supplementation with vitamin A to assure vitamin A adequacy; this finding suggests that the hepatic production of RBP was rate-limiting during inflammation (Stephensen *et al.*, 2002a), and is consistent with the animal studies discussed above. Even in the general US population, plasma retinol and CRP were significantly inversely correlated as shown in an analysis of data collected in the National Health and Nutrition Examination Survey (NHANES), and it was suggested that low plasma retinol might be misclassified as being due to a nutritional deficiency when the actual cause is inflammation (Stephensen and Gildengordin, 2000). From a combined analysis of 15 studies in children in which retinol and acute-phase proteins were measured, it was estimated that plasma retinol is reduced for individuals with infection, as compared with healthy individuals, by 13%, 24% and 11% in the

incubation, early convalescent and late convalescent stages of subclinical infection, respectively (Thurnham *et al.*, 2003). Additionally, in studies of children with diarrhea and fever, retinol was lost in urine (Mitra *et al.*, 2002), suggesting that irreversible loss of retinol as well as its redistribution from plasma into tissues, as shown in animal studies, may be increased by infection or inflammation.

It is unknown whether various types of chronic low-grade inflammation, such as in conditions of arthritis or chronic mild infection, are severe enough to alter plasma retinol or tissue retinoid status, but these areas are important for further research. The finding of a negative association between plasma retinol and CRP levels observed in NHANES, a nationally representative survey of the apparently healthy US population, suggests this could be a possibility.

8.4.3 Effects of inflammation on enzymes involved in retinoid metabolism

Inflammation affects the expression of at least some of the genes involved in maintaining retinoid homeostasis. Whereas the expression of CYP26A1 and CYP26B1, genes that encode RA-4 hydroxylase activity (Ross and Zolfaghari, 2011), was markedly elevated in the liver of rats treated with RA, co-treatment with LPS along with RA nearly completely abrogated the increase due to RA (Zolfaghari *et al.*, 2007). This reduction in CYP26A1 and CYP26B1 expression suggests that inflammation could impair RA catabolism, at least in liver, which may be a biological mechanism for preserving RA. Such a change could also potentially result in a build-up of excessive RA during use of RA for therapy. In other studies in which ^3H -RA was used as a tracer to monitor the hepatic uptake of RA and its conversion to polar metabolites in vitamin A-marginal rats, LPS-induced inflammation lowered the amount of RA cleared into liver, while increasing RA uptake in the small intestine (Cifelli and Ross, 2006).

8.5 Pro-inflammatory and anti-inflammatory aspects of retinoids in the skin

Due to their ability to promote cell differentiation and reduce cell proliferation, retinoids were first tested as far back as the 1970s for their ability to treat mild to severe skin disorders. As noted by Roos *et al.* (1998), the importance of retinoids in dermatology dates back even further to Wolbach and Howe in 1925, who identified abnormal keratinization of the skin in VAD animals (Wolbach and Howe, 1925). When RA or retinoid analogs are used topically, they often initially cause a reddening and mild irritation of the skin indicative of local inflammation (Fisher *et al.*, 1991). It seems these effects may be relatively non-specific irritant effects because similar changes occurred in subjects treated with sodium lauryl sulfate (Fisher *et al.*, 1991). As discussed further below, retinoid treatments that reduce the concentrations, modify the delivery or combine retinoids with other therapeutic agents are currently in use. Retinoids have also been delivered systemically. The use of systemic retinoids, while effective, is now highly regulated due to

longstanding concerns about retinoid-induced teratogenesis (Soprano and Soprano, 1995; Ganceviciene and Zouboulis, 2010). Besides being used for skin disorders, retinoids have also been studied experimentally for their potential anti-inflammatory activity in models of skin wound healing and repair.

8.5.1 Retinoid treatments

All-*trans*-RA [tretinoin (Retin-A)], was released in the USA in 1971, and has subsequently been used for treating acne and photoaging. Tazarotene, released in, 1997, shows antipsoriatic activity and is approved for acne treatment (Del Rosso, 2002). The most potent inhibitor of sebum production, which is a key component in the pathophysiology of acne vulgaris, is the 13-*cis* isomer of RA (isotretinoin; Accutane), often administered orally (Ganceviciene and Zouboulis, 2010). However, 13-*cis*-RA does not bind tightly to CRABPs or transactivate RARs, and thus its mode of action *in vivo* is unclear. However, in cultured SEB-1 sebocytes 13-*cis*-RA but not all-*trans*-RA or 9-*cis*-RA induced apoptosis (Nelson *et al.*, 2006). Whereas retinoids are often considered potent agents of cell differentiation, it is believed that 13-*cis*-RA causes the sebaceous glands to dedifferentiate and stop producing sebum (Saurat, 1997).

Topically applied retinoids exhibit therapeutic effects on photoaged skin. A Cochrane review of clinical trials concluded that topical tretinoin compared to placebo improves the appearance of mild to moderate photodamage on the face and forearms, in the short term (Samuel *et al.*, 2005). Topical RA preparations resulted in increased epidermal thickening due to increased keratinocyte proliferation, expansion of intercellular spaces and compaction of the epidermal barrier, while numerous molecular changes include the induction of the genes for CRBP, CRABP-II and CYP26 (Roos *et al.*, 1998; Fisher *et al.*, 1991).

It has been proposed that retinol may be a more efficient and natural way to deliver RA to the correct subcellular location within skin cells, compared to direct treatment with RA. In clinical studies of photodamaged skin, 0.1% retinol used topically resulted in improvements in all major aging signs, and increased keratinocyte proliferation and epidermal thickness (Bellemère *et al.*, 2009).

Combination treatments have also been tested. In the treatment of inflammatory acne lesions, topical retinoids (tretinoin, adapalene, tazarotene) combined with benzoyl peroxide have reduced formation of microcomedones and inflammatory papules and pustules (Del Rosso, 2002). A systematic review on treatments for plaque-type psoriasis concluded that single-agent therapy with retinoids was not effective, but retinoids combined with phototherapy appeared to be highly effective (Sbidian *et al.*, 2011).

8.5.2 Retinol metabolism in the skin

Retinol is metabolized in human skin to retinyl esters, 3,4-didehydroretinol and small quantities of all-*trans*-RA and 3,4-didehydroRA (Roos *et al.*, 1998). The minimal accumulation of all-*trans*-RA may be due to induction of CYP26 whose

retinoic acid-4-hydroxylase activity exclusively metabolizes all-*trans*-RA. Inflammatory conditions such as psoriasis are associated with alterations in the levels of CRABP-I and CRABP-II, suggesting that retinoid metabolism could be altered in such diseases.

8.5.3 Other aspects of retinoids in skin health

Retinoids also may affect the process of skin wound healing. Tretinoin and adapalene administered to rats with full thickness dermal wounds was reported to enhance collagen production, angiogenesis and granulation tissue formation (Basak *et al.*, 2002). In a pilot study of human skin in five patients with long-standing leg ulcerations, topical application of 0.05% tretinoin for 10 minutes per day increased the granulation response and appeared promising as a clinical modality (Paquette *et al.*, 2001).

8.6 Vitamin A and immunity

Poor vitamin A status has been associated with increased occurrence and severity of infectious disease (reviewed in Stephensen, 2001; Villamor and Fawzi, 2005). The innate and adaptive immune systems, which receive input from many innate immune factors, are dysregulated by VAD and, conversely, are responsive to vitamin A and RA as shown both *in vitro* and *in vivo*. Recent investigations have focused on understanding how cytokines and RA interact to alter the balance of T cells that, in turn, regulate various types of immune responses. Several concepts have emerged: (i) that VA and RA promote Th2 cell differentiation while restraining Th1 cell differentiation; (ii) that RA is an important factor in the differentiation of T-regulatory (Treg) cells, especially in the intestine, with implications for mucosal homeostasis and prevention of intestinal inflammatory disorders; (iii) that RA regulates lymphocyte homing to the gut, and that dendritic cells (DC), particularly in the intestine, are an important source of RA for this regulation. The latter finding is based on limited data and will be discussed critically as an area that requires further study. Additionally, (iv) recent evidence suggests novel mechanisms through which RA may contribute to the antiviral response necessary for defense against measles virus infection. Each of these areas has important clinical and/or public health implications. Lastly, (v) adequate vitamin A is also essential for a normal response to immunization and vaccination, which, while not reviewed here, has been the subject of several recent reviews (Stephensen, 2001; Villamor and Fawzi, 2005; Ross *et al.*, 2009, 2011a).

8.6.1 Vitamin A and RA promote Th2 and restrain Th1 cell differentiation

The use of rodent models has helped define the mechanisms by which vitamin A status alters the immune response. Generally, VAD causes a weakened Th2

response, characterized by low IL-4 and IL-5 production and reduced immunoglobulin (Ig) production, at least for certain Ig classes, and an unaltered or heightened Th1 response, associated with decreased and/or dysregulated populations of circulating lymphocytes including CD4⁺ and CD8⁺ T cells (Smith *et al.*, 1987; Zhao and Ross, 1995; Ross, 1996; Iwata *et al.*, 2004). The weakened Th2 response in VAD mice was associated with lower numbers of IL-5-secreting cells, and the pronounced Th1 response with an increased rate of IFN- γ production and antigen presenting cell (APC) activity (Cantorna *et al.*, 1994). Antibody responses in VAD models, reviewed previously (Ross, 1996), have generally resulted in lower titers of IgG antibodies. In APCs and/or T cells, RA decreased Th1-related cytokines and receptors including IL-12, IL-12 β 2, and IFN- γ , as well as the transcription factor T-bet, a master regulatory factor for the Th1 lineage (Ma *et al.*, 2005). Conversely, Th2-related factors including expression of IL-4, IL-5, GATA-3, a master regulatory factor for the Th2 lineage, and c-maf, a transcription factor necessary for the production of the anti-inflammatory cytokine IL-10, were all upregulated by RA (Stephensen *et al.*, 2002b; Iwata *et al.*, 2003; Ma *et al.*, 2005) or related retinoids (Kang *et al.*, 2000; Stephensen *et al.*, 2002b). Whether RXR ligands have a role is less clear (see Stephensen *et al.*, 2002b; Iwata *et al.*, 2003), but all-*trans*-RA has been consistently demonstrated to promote Th2 differentiation and/or inhibit Th1 differentiation in models relevant to infectious disease. When activated splenocytes were stimulated with IFN- γ or IL-4, cytokines that promote Th1 or Th2 differentiation, respectively, the addition of all-*trans*-RA inhibited subsequent IFN- γ production and promoted a five-fold increase in IL-4 production (Hoag *et al.*, 2002). It was further elucidated that RA was also affecting APCs, as APCs activated with transgenic peptide and treated with RA displayed pronounced IL-4 production (Hoag *et al.*, 2002).

RA also promotes the Th2 response by upregulating the expression of the costimulatory molecule, CD86, and the natural killer T (NKT) cell population, which was correlated to increased secretion of IL-4, Th2 development and increased production of IgG1 antibodies following immunization (Ma *et al.*, 2005).

8.6.2 Other polarized cell phenotypes

Recent studies have extended the Th1/Th2 concept to macrophages and monocytic cells. In lung alveolar macrophages from *Ascaris*-infected pigs treated *ex vivo* with IL-4 and/or RA, addition of IL-4 increased the expression of mRNA for the chemokines CCL11, CCL17, CCL22 and CCL26 (eotaxin 3), most consistently associated with late-phase tissue eosinophilia, while RA increased the basal and IL-4-stimulated expression of CCL17 and CCL22 (Dawson *et al.*, 2009). These results suggest that RA aids IL-4 in polarizing macrophages towards the alternatively-activated M2 phenotype, related to Th2 responses *in vivo*. In the human monocytic THP-1 cell line, RA strongly induced the expression of CD1d, an MHC class I-like molecule that binds and presents glycolipid antigens to T cells, especially NKT cells (Chen and Ross, 2007). When stimulated, NKT cells rapidly

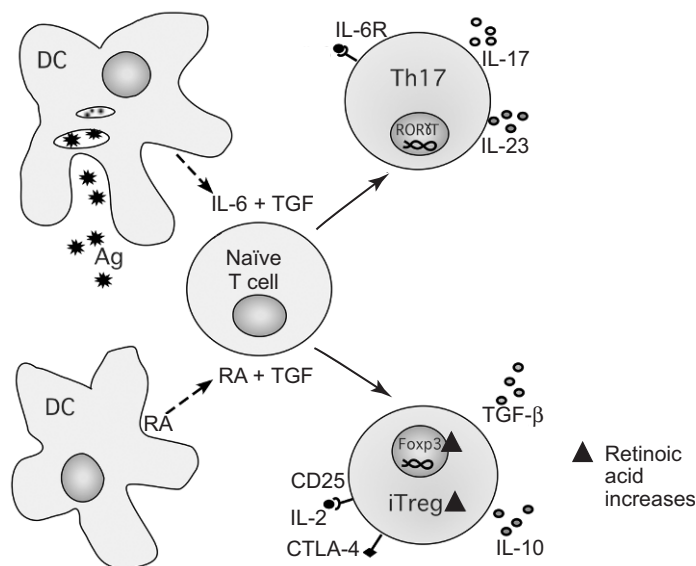


Fig. 8.3 Schematic of differentiation of a naïve CD4 T cell into Th17 cells under the influence of IL-6 and TGF- β or, alternatively, into induced T-regulatory cells (iT-reg) under the influence of RA and TGF- β . Th17 cells generally fight infection, recruit neutrophils and express inflammatory cytokines, while iTreg cells generally are immunosuppressive and helpful in maintaining oral tolerance and homeostasis in the intestine (Mucida *et al.*, 2009; Weaver and Hatton, 2009). RA can suppress the Th17 population (Xiao *et al.*, 2008). Moreover, these cell populations are not static but instead are responsive to the cytokine milieu, such that Foxp3-positive iTreg cells under the influence of IL-6 may gain properties of Th17 cells (Weaver and Hatton, 2009; Ziegler and Buckner, 2009; Zhou *et al.*, 2008). In the nucleus of Th17 cells, ROR γ t is expressed and Foxp3 is suppressed, while in the nucleus of iTreg cells, Foxp3 is induced in the presence of RA.

secrete both IFN- γ and IL-4. Experimentally, mouse spleen cells treated with the CD1d ligand α -galactosylceramide (α -GalCer) produced higher levels of both IFN- γ and IL-4 but, in the presence of RA, the production of IFN- γ was reduced and IL-4 further increased (Chen and Ross, 2007), also consistent with the promotion of Th2 immunity as demonstrated *in vivo* (Chen *et al.*, 2011).

8.6.3 RA promotes T-regulatory and suppresses Th17 lineage development

Another subset of T helper cells, Treg cells, control inflammation by secreting the anti-inflammatory cytokine IL-10. Treg cells are considered essential for intestinal homeostasis and tolerance. In contrast, Th17 cells protect the host from infections by releasing the pro-inflammatory cytokines IL-17 and IL-23, and are crucial in the response to pathogenic microbes (Ivanov *et al.*, 2007). The mechanisms responsi-

ble for initiating the differentiation of Treg and Th17 cells and maintaining the balance between them are understood to be important but complex, and much remains to be elucidated (Mucida *et al.*, 2007; Zhou *et al.*, 2008; Ziegler and Buckner, 2009). Treg cells comprise both natural Treg cells that develop from naïve T cells in the thymus (nTreg), and those that develop transiently in the periphery in response to antigen stimulation, inducible Treg (iTreg) (Mucida *et al.*, 2009). In the presence of TGF- β , an abundant cytokine in the intestinal mucosa, RA suppresses Th2 and favors Treg differentiation (Takaki *et al.*, 2008). A schematic of this process is shown in Fig. 8.3.

Additional molecules necessary for iTreg development include CTLA-4, the α -chain of the IL-2 receptor (CD25) and Foxp3, a master regulatory transcription factor for the iTreg lineage (Chen *et al.*, 2003). RA in combination with TGF- β induces Foxp3 expression by signaling through Smad3, and Foxp3 downregulates Smad7, an inhibitory protein of Treg differentiation (Fantini *et al.*, 2004; Xiao *et al.*, 2008). Foxp3 is upregulated by the transcription factors NFAT and Smad3 that associate with an enhancer site upstream of the Foxp3 promoter (von Boehmer and Nolting, 2008). Once Foxp3 is expressed, it associates with the NFAT2 promoter to further the differentiation of iTreg cells, and to block IL-2 secretion, which aids in other T cell-lineage commitment (Torgerson *et al.*, 2009). Additionally, STAT6 functions to negatively regulate Treg conversion by direct association with the Foxp3 promoter, a mechanism that is reversed by RAR- α signaling (Elias *et al.*, 2008). RA and TGF- β signaling decreased STAT6 association and increased histone acetylation on the Foxp3 promoter (Takaki *et al.*, 2008).

Several cytokines and growth hormone signaling molecules control the balance between Treg and Th17 differentiation. Interestingly, TGF- β is necessary for both Treg and Th17 cell differentiation as picomolar concentrations of TGF- β allow synergy between IL-6 and IL-21 to induce IL-23r and IL-17, thus promoting Th17 lineage commitment, but higher concentrations of TGF- β repress IL-23r expression in favor of Foxp3⁺ Treg differentiation (Ivanov *et al.*, 2007; Zhou *et al.*, 2008). IL-6, IL-21, IL-23, TGF- β and ROR γ T, a master regulatory factor for the Th17 lineage, promote Th17 differentiation, whereas IL-2, IL-4, IFN- γ and IL-27 inhibit differentiation (Ivanov *et al.*, 2007). IL-6 primes naïve T cells for a Th17 fate and STAT1/3 signaling upregulates IL-23 expression, which is necessary for later stages of Th17 development (Ivanov *et al.*, 2007). Both TGF- β and Foxp3 antagonize the synthesis of ROR γ T by directly binding to the promoter and inhibiting transcription (Ivanov *et al.*, 2007; Zhou *et al.*, 2008). RA plays an important role in controlling the function of TGF- β by inhibiting IL-6-mediated differentiation of pro-inflammatory Th17 cells and enhancing Treg cells (Mucida *et al.*, 2007). In brief, RA and TGF- β appear to function cooperatively to inhibit Th17 differentiation by upregulating Foxp3, which directly inhibits ROR γ T, as well as by decreasing cytokine signaling that promotes Th17 development (Ivanov *et al.*, 2007; Takaki *et al.*, 2008; Xiao *et al.*, 2008).

8.6.4 RA regulates lymphocyte homing to the gut

Mice rendered VAD by feeding a vitamin A-deficient diet were shown to have markedly fewer CD4 and CD8 T cells in the lamina propria (LP), but nearly normal numbers in nearby intestinal lymph nodes (Iwata *et al.*, 2004), suggesting that T cell migration into the LP was impaired in the VAD state. As illustrated in Fig. 8.4, RA is thought to mediate T cell homing to the intestine because of its ability to upregulate chemokine and integrin molecules that are required for homing on T lymphocytes of the gut-associated lymphoid tissues (GALT, e.g. mesenteric lymph nodes (LNs), Peyer's patch) and gut endothelial cells. Oral antigen-induced CD103⁺ GALT DCs were shown to stimulate CD8⁺ T cells to adopt a gut-homing phenotype (Johansson-Lindbom *et al.*, 2005). The chemokine CCL25 and the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) are expressed on endothelial cells of the LP and small intestine. The receptor for CCL25, CCR9, as well as $\alpha_4\beta_7$ integrin, which has a high affinity for MAdCAM-1, are expressed on GALT T cells which upon upregulation, promotes T cell homing to the small intestine (Papadakis *et al.*, 2000; Johansson-Lindbom *et al.*, 2005). Stimulation of activated CD8⁺ T cells with DCs from various LNs including the mesenteric LN upregulated CCR9 and $\alpha_4\beta_7$ integrin levels (Svensson *et al.*, 2002; Mora *et al.*, 2003; Iwata *et al.*, 2004; Johansson-Lindbom *et al.*, 2005), while additional chemokine signaling molecules such as CCR7 also promoted gut homing (Johansson-Lindbom *et al.*, 2005). Treatment with all-*trans*-RA upregulated CCR9 and $\alpha_4\beta_7$ integrin on CD8⁺ T cells in the GALT, and CCL25 and MAdCAM-1 expression on the intestinal epithelium (Edele *et al.*, 2008; Svensson *et al.*, 2008; Eksteen *et al.*, 2009).

Several reports have described a subset of CD103⁺ CCR7⁺ DCs from mesenteric and peripheral LNs that have the capability to synthesize RA *de novo* which, in turn, is proposed to upregulate T cell homing molecules in the GALT and thereby T cell homing to the small intestine in response to appropriate TCR stimulation (Iwata *et al.*, 2004; Mucida *et al.*, 2009). When co-cultured with activated T cells, CD103⁺ DCs from the mesenteric and other LNs increased expression of

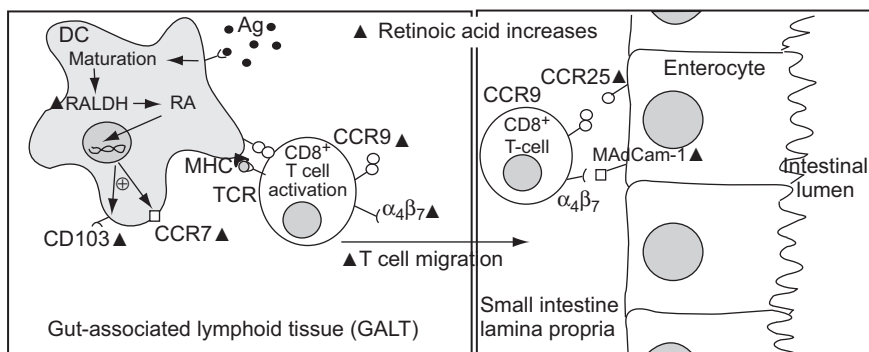


Fig. 8.4 Overview of mucosal T cell activation and homing to the lamina propria (LP) after antigen activation. Triangles mark processes shown to be increased in RA-treated cells in culture or by RA *in vivo*. Ag, antigen.

retinaldehyde dehydrogenase Aldh1a2 (RALDH2) mRNA (Iwata *et al.*, 2004; Coombes *et al.*, 2007). Furthermore, VAD mice have decreased expression of several aldehyde dehydrogenase genes in CD103⁺ DCs, decreased $\alpha_4\beta_7$ expression on T cells in lymphoid organs, and reduced T lymphocyte homing to the LP (Iwata *et al.*, 2004). One study detected ³H-RA production from *ex vivo* cultured Peyer's patch and mesenteric LN DCs after the addition of ³H-retinol (Iwata *et al.*, 2004). Citral, a retinal dehydrogenase inhibitor, did not alter $\alpha_4\beta_7$ or CCR9 expression in one investigation, but the upregulation of T cell homing markers was lost after citral treatment in another report (Iwata *et al.*, 2004; Saurer *et al.*, 2007). RAR antagonists decreased expression of $\alpha_4\beta_7$ and CCR9 and subsequent T cell homing (Iwata *et al.*, 2004; Saurer *et al.*, 2007; Elgueta *et al.*, 2008).

Although it is clear that activated GALT DCs upregulate genes nominally involved in RA synthesis, the notion that GALT DC-synthesized RA regulates T cell migration is still based on mostly circumstantial evidence. Of three Aldh1 family genes (Aldh1a1, Aldh1a2 and Aldh1a3), data relating Aldh1a2 to RA production are most convincing, based on a variety of studies, but members of this gene family often have multiple substrates, and detection reagents such as Aldefluor® are not specific for detecting retinaldehyde nor are enzyme inhibitors such as citral and diethylaminobenzaldehyde (DEAB) specific for inhibiting only RA production. Potentially, other cells such as epithelial cells could also be a source of RA in the LP, as could RA taken up from the circulation. Further research is needed to establish the relationship between dietary retinol and intestinal RA levels and to confirm the roles of various immune cell types in producing RA.

8.6.5 Sources of RA

If RA is being synthesized by DCs of the GALT and in the LP, what intestinal function(s) is RA signaling regulating? It has been observed that LP DCs induce the Treg phenotype after the addition of TGF- β and RA (Mucida *et al.*, 2007; Sun *et al.*, 2007). A substantial population of Foxp3⁺ CD4⁺ T cells exists in the LP with Foxp3⁺ Treg cells comprising 20% of the CD4⁺ T cells in the small intestine and 40% in the large intestine (Ivanov *et al.*, 2007). Additionally, Treg induction coincided with expression of RALDH, IL-10 and TGF- β by intestinal DCs, and the expression of these factors was dependent on β -catenin and TLR2 signaling (Manicassamy *et al.*, 2009, 2010). Therefore, the mechanisms of RA-mediated iTreg induction are likely to be very complex. Nonetheless, RA appears to be an important modulator of the delicate balance that must be maintained between the pro-inflammatory Th1 and Th17 responses that protect the host from infection, and the anti-inflammatory iTreg response which functions to police inflammatory reactions in order to neutralize the threat of autoimmune disorders such as the inflammatory bowel diseases Crohn's disease and ulcerative colitis. Adding further complexity to this picture, a recent study in mice showed that RA, in the presence of DCs transgenic for IL-15, a cytokine that is often elevated in the intestinal mucosa of persons with Crohn's disease, acted in a pro-inflammatory manner and enhanced Th1 cell IFN- γ production (DePaolo *et al.*, 2011). Therefore

an effect of RA opposite to that expected might result if the cytokine milieu is altered, for example as by a high level of IL-15.

8.6.6 Retinoids inhibit measles virus replication

Vitamin A supplementation has been used since the late 1980s to treat measles virus (MV) as it decreases morbidity and mortality (D'Souza and D'Souza, 2002). MV is a negatively-charged ssRNA virus belonging to the *Paramyxoviridae* family and is spread through respiratory droplets, first infecting lymphocytes and monocytes of the respiratory tract and then disseminating throughout the host (Rodeheffer *et al.*, 2007). Recent *in vitro* work (Trottier *et al.*, 2008) demonstrated that vitamin A signaling through RAR inhibits MV replication at lower multiplicity of infections, or earlier during infection when there are fewer viral transcripts. The inhibition of MV replication by vitamin A was independent of other previously hypothesized mechanisms, i.e. promotion of cell differentiation, proliferation and viability – all functions that maintain epithelial barrier integrity (Stephensen, 2001; Trottier *et al.*, 2008). One means by which MV spreads is by abrogation of type I IFN production, while retinoids have the ability to induce the IFN-stimulated genes such as IRF7 and STAT1 in bystander cells not infected by MV, thus providing protection from further rounds of virus replication (Trottier *et al.*, 2009).

There is no known animal model to study MV, but recently, the ferret infected with canine distemper virus (CDV) has been used as a comparable animal model (Rodeheffer *et al.*, 2007). CDV is also a negatively-charged ssRNA paramyxovirus, with a similar disease progression as MV. In agreement with *in vitro* studies of retinoids and MV, CDV was less severe in ferrets with an adequate vitamin A status produced by high-dose retinol supplementation, while VAD ferrets displayed exacerbated CDV severity and increased mortality (Rodeheffer *et al.*, 2007).

All-*trans*-RA and MV induced RIG-I gene transcription. RIG-I is an RNA helicase that detects viral transcripts, specifically 5'-triphosphate ssRNA, in the cytoplasm during viral replication and induces type I IFN production (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). Interestingly, RIG-I-deficient mice were unable to respond to other paramyxoviruses (Pichlmair *et al.*, 2006). Future investigations will likely determine if vitamin A status aids in RIG-I function and perhaps define other mechanisms by which vitamin A ameliorates disease severity. Altogether, retinoids are an effective treatment for MV in part by inhibiting replication in an RAR-dependent manner due to upregulating interferon-stimulated genes in uninfected neighbor cells (Trottier *et al.*, 2008, 2009).

However, the effect of vitamin A may be both context- and virus-specific. In a study of mice fed a diet high in vitamin A and challenged intranasally with influenza A virus, a pronounced Th2 response was observed, marked by increased IgA and IL-10 secretion and decreased IgG and IFN- γ production (Cui *et al.*, 2000). Since viral infections are generally resolved in a Th1-mediated fashion, the augmentation of the Th2 response by vitamin A may provide one such explanation as to why vitamin A supplementation may not aid in the resolution of viral pneumonia (Cui *et al.*, 2000).

8.7 Future trends

Vitamin A and RA have complex effects on many processes, and it is not possible to categorically classify retinoids as either pro- or anti-inflammatory. The multiple abnormalities in the immune system that are observed in VAD animal models, and inferred from human studies as well, all indicate that an adequate intake of dietary vitamin A is essential for a normally developed and properly functioning immune system. As a broad generalization, it can be said that retinoids often promote reactions that are anti-inflammatory, such as by favoring production of Th2 vs Th1, and Treg vs Th17 cells, and the cytokines these cells produce. However, to combat certain infections, Th1 and Th17 responses are essential, and thus an excess of vitamin A and/or RA could be unfavorable in these circumstances. As the preceding section has indicated, the context of the immune response – the location of infection or inflammatory response, type of infectious agent and type of immune response it naturally triggers – may be critical to whether or not vitamin A and/or RA improves outcome. This poses challenges for using high-dose vitamin A supplementation in public health programs, as the health status of the recipient is generally not known in detail, and for using RA as a therapeutic agent when a treatment meant for one condition could inadvertently interfere with or misdirect the response to another condition. Overall, an adequate dietary intake of vitamin A is the best current practice for promoting optimal health. Further research is needed to better define the roles of RA in viral and microbial disease models that are relevant to human health.

8.8 Acknowledgments

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9

Vitamin D and the immune system

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Abstract: This chapter discusses the role of vitamin D in the regulation of the immune system. The epidemiology, biology and physiology of vitamin D in humans is described, followed by a detailed exposition of the actions of 1,25(OH)₂-vitamin D on immune cells. The chapter concludes that vitamin D appears to be important in immune function, but that at this time there is insufficient evidence on which to base clinical recommendations regarding supplementation.

Key words: vitamin D, calcitriol, autoimmunity, allergy, T cells.

9.1 Introduction

Vitamin D is a secosteroid, synthesized in the skin via the action of sunlight. It plays a critical role in bone metabolism and skeletal growth (Holick, 2003). Around 95% of vitamin D is acquired via photosynthesis in the skin, with the minority coming from the diet in most humans. There are two forms obtained from dietary sources: ergocalciferol (D₂), from plants, and cholecalciferol (D₃), from animals; the latter is mainly found in oily fish and fortified margarines and breakfast cereals and is also the form produced endogenously (Holick and Garabedian, 2006).

9.1.1 Biosynthesis of the active form of vitamin D

Vitamin D is synthesized in the skin from the action of sunlight (wavelengths 290–315 nm) on 7-dehydrocholesterol, causing its conversion to pre-vitamin D₃.

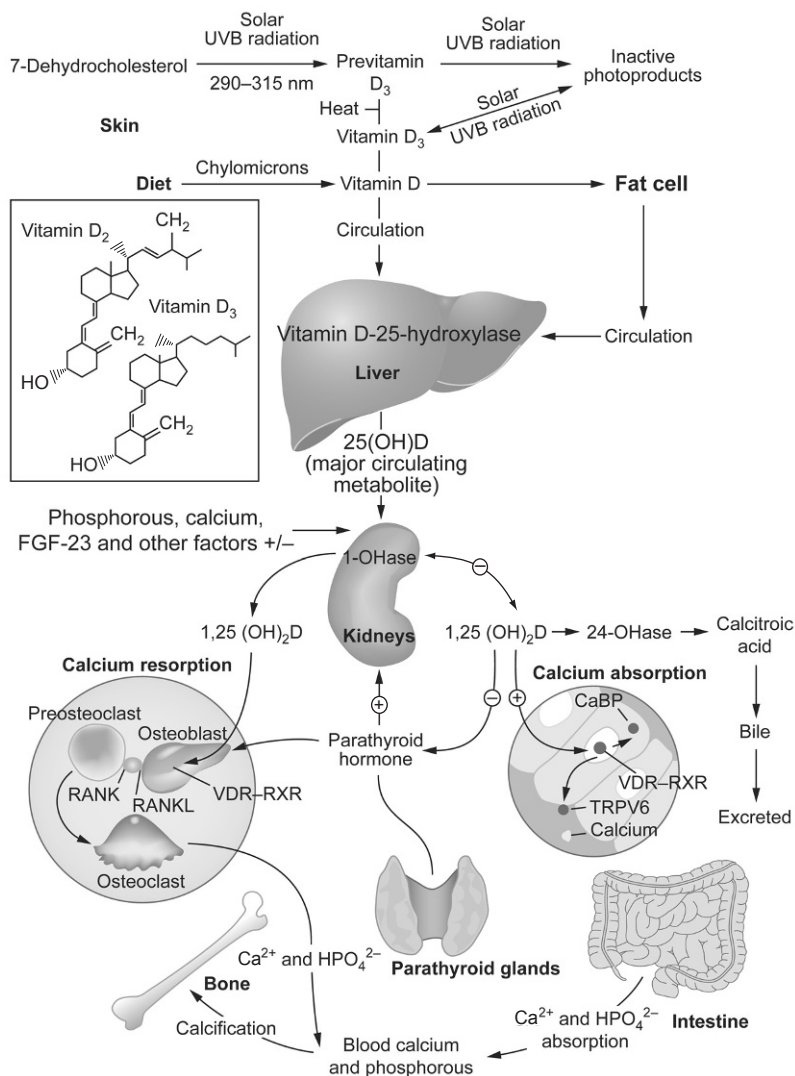


Fig. 9.1 Synthesis and metabolism of vitamin D and its role in the regulation of calcium, phosphorus and bone metabolism. CaBP, calbindin; FGF-23, fibroblast growth factor 23; TRPV6, transient receptor potential cation channel; VDR-RXR, vitamin D receptor-retinoid-X receptor heterodimer. Reproduced with permission from Holick (2007).

(Holick, 2003, 2004a) (Fig. 9.1). Following its formation, pre-vitamin D₃ undergoes membrane-enhanced temperature-dependent isomerization to vitamin D₃ (Holick, 2003), which is translocated into the circulation, where it is carried by vitamin D-binding protein (DBP) (Holick, 2004a). In most situations, the main predictor of vitamin D synthesis in the skin is the level of exposure to appropriate

wavelength UVB radiation. The total amount of energy accrued from sunlight is dependent on duration and extent of skin exposure, but also on latitude and season. Thus, being dark-skinned, covered or using sun-block with a factor higher than eight reduces cutaneous formation of vitamin D (Holick and Garabedian, 2006). At latitudes of 48.5° (Paris, France) and above, the skin is unable to form vitamin D between the months of October through to March (Holick, 2003). This results in a seasonal variation in vitamin D concentrations in northern latitudes, with a peak over the summer and a trough in the winter (Holick, 2004a).

Circulating vitamin D is converted in the liver to 25(OH)-vitamin D (calcidiol), which is the main circulating form. This step, involving the cytochrome P450 system, is not rate limiting: greater photosynthesis of vitamin D in the skin will lead to an increase in serum 25(OH)-vitamin D (DeLuca, 2004; Holick, 2004a). Excess 25(OH)-vitamin D is converted to 24,25(OH)-vitamin D which is thought to be relatively metabolically inactive (Holick, 2004a). The 25-(OH)-vitamin D-DBP complex enters renal tubule cells by membrane-bound megalin transport, where 25(OH)-vitamin D is converted to 1,25(OH)₂-vitamin D (calcitriol) by the enzyme 1- α -hydroxylase, regulated by parathyroid hormone (PTH) (DeLuca, 2004). Although the kidney is the primary site for conversion of circulating 25(OH)-vitamin D, there is evidence for 1- α -hydroxylase expression in a wide range of cells and tissues (Sharma, 2000; Holick, 2003, 2004b), and this may contribute to local paracrine signalling, rather than having a role in calcium homeostasis (Holick and Garabedian, 2006).

9.1.2 Action of vitamin D

1,25(OH)₂-vitamin D is around 1000 times more potent than 25(OH)-vitamin D, but is present in the circulation at much lower concentrations (Holick, 2003). However, it seems to be the predominant active form of the vitamin, acting at a nuclear receptor site. According to its classic mechanism of action, 1,25(OH)₂-vitamin D binds to a cytoplasmic receptor, the vitamin D receptor (VDR), and this complex travels into the nucleus, where it dimerizes with RXR (Haussler *et al.*, 1998; Barsony, 2005). This complex binds to the vitamin D response elements (VDRE) on DNA and this induces the binding of several initiation factors, resulting in transcription of the vitamin D responsive gene (Haussler *et al.*, 1998) (Fig. 9.2).

The major physiological function of vitamin D is to maintain calcium concentrations within the appropriate range in the circulation, mainly by regulating absorption from the small intestine. Thus 1,25(OH)₂-vitamin D facilitates calcium entry from the small intestine lumen by inducing the epithelial calcium channel. It also induces several other proteins in the intestinal epithelial cells, including calcium-binding protein (calbindin D9K), alkaline phosphatase, low-affinity Ca ATPase, brush border actin and calmodulin (Bouillon, 2001). There appears to be a biphasic response with a rapid initial increase in calcium flux over a couple of hours, followed by a more sustained increase that begins at 12 hours, suggesting that 1,25(OH)₂-vitamin D may have a rapid membrane effect, followed by a slower action on gene expression (Bouillon, 2001; Christakos *et al.*, 2003).

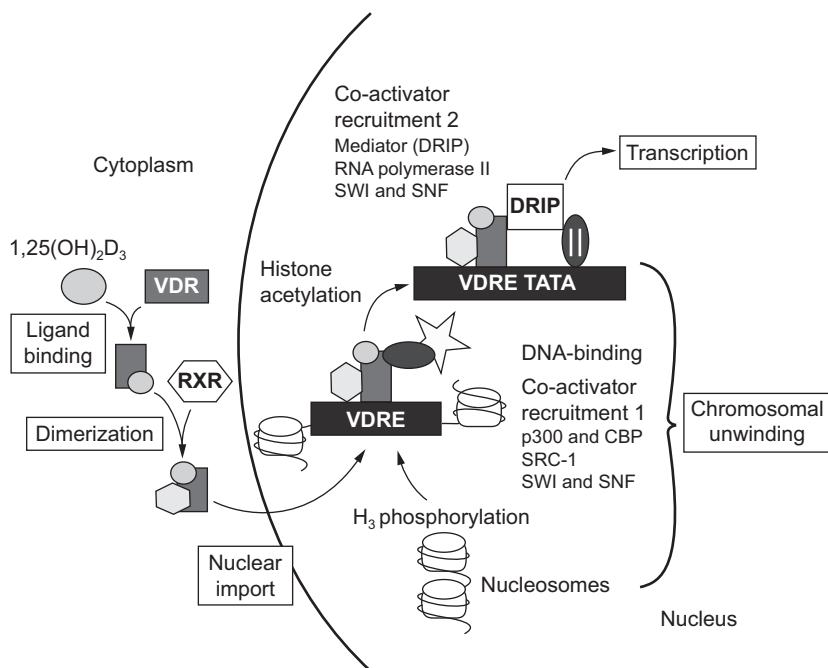


Fig. 9.2 Regulation of gene transcription through the nuclear vitamin D receptor (VDR). Reproduced with permission from Fleet *et al.* (2004).

The net result of vitamin D action at the intestinal level is an increase in calcium and phosphorus absorption. In the vitamin D-deficient state, no more than 10–15% of dietary calcium and 60% of phosphorus is absorbed but, with adequate vitamin D, this fractional absorption may rise to 30–40% for calcium and 80% for phosphorus (Bouillon, 2001; Christakos *et al.*, 2003). The main role of $1,25(\text{OH})_2$ -vitamin D in bone mineralization is by maintenance of the calcium $\times$ phosphorus product in the blood. If there is inadequate dietary calcium then the $1,25(\text{OH})_2$ -vitamin D–VDR interaction results in RANKL production by osteoblasts, causing pre-osteoclasts to differentiate into osteoclasts and to resorb bone, thus increasing the blood calcium and phosphorus concentrations (Khosla, 2001; DeLuca, 2004) (Fig. 9.1).

9.1.3 Control of vitamin D axis by PTH

The parathyroid chief cells contain a VDR, and $1,25(\text{OH})_2$ -vitamin D acts on this to decrease PTH synthesis and secretion. The parathyroid glands also contain a calcium-sensing receptor (CaSR), which acts to maintain blood calcium concentration within set limits (Holick and Garabedian, 2006). Thus decreased calcium concentration leads to an increase in PTH concentration in the blood. Parathyroid hormone acts to increase conversion of 25 to $1,25(\text{OH})_2$ -vitamin D by stimulating the renal 1α -hydroxylase, via renal wasting of phosphorus, has a direct effect on

calcium and phosphorus resorption from bone (Holick and Garabedian, 2006), and increases renal retention of calcium.

9.2 Food sources, recommended intakes and epidemiology of deficiency

The most effective sources of vitamin D are oily fish (for example salmon, mackerel) and fortified foods such as margarine and breakfast cereal, with few other foods containing significant amounts. The quantity of vitamin D derived from fish is usually modest: wild salmon may contain around 400 iu per 3.5 oz (Holick, 2004a). There is much controversy over the recommended daily intake of vitamin D. Early guidance has suggested 200 iu per day for children and adults up to 50 years old and 400–600 iu for older adults (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1999). However, humans have probably evolved to synthesize much higher levels of vitamin D in the skin: 30 minutes exposure at midday in the summer sun at a southerly latitude in a bathing suit might release around 20 000 iu into the circulation within 24 hours in white persons (Adams *et al.*, 1982). The evidence base from which to derive guidance on normal levels and the doses of supplementation required to achieve them is scant. There is controversy regarding the likely required daily intake for health: dosing studies have suggested that supplementation with 200–400 iu per day is unlikely to maintain levels of 25(OH)-vitamin D over winter months (Heaney *et al.*, 2003), and higher doses are likely to be required in those who are frankly deficient (Dawson-Hughes *et al.*, 2005). Recent guidance from the Institute of Medicine (IOM, 2010) has attempted to review the totality of the evidence base, and has recommended that an Estimated Average Requirement (EAR) of 400 iu/day and a Recommended Dietary Allowance (RDA) of between 600 and 800 iu/day is appropriate for the majority of the US population.

9.2.1 Epidemiology of vitamin D deficiency

There are very few population-based estimates of vitamin D levels. In one cohort in Southampton, composed of white Caucasian young women, 31% had concentrations of circulating 25(OH)-vitamin D lower than 50 nmol/l and 18% less than 25 nmol/l (Javaid *et al.*, 2006). In Asian cohorts in the northern hemisphere the burden is even higher (Roy *et al.*, 2007), possibly reaching 90% or greater. As the main source of vitamin D is synthesis in the skin under the influence of UVB radiation from sunlight exposure, ethnicity (dark skin), covering and northerly latitudes (as in the UK) are all major risk factors for insufficiency (Holick, 2004a). Thus in a study of south-Asian women in the north of England, many of whom were of child-bearing age, 94% had circulating levels of 25(OH)-vitamin D < 37.5 nmol/l and 26% < 12.5 nmol/l (Roy *et al.*, 2007); a survey of the UK population revealed low levels of 25(OH)-vitamin D in 50% of the people (Hypponen *et al.*, 2007). A US study of a population representative of the national demographic

distribution revealed that 80% of black pregnant women had levels less than 50 nmol/l; the figures for Hispanic and white pregnant women were 45% and 13%, respectively, with levels less than 50 nmol/l (Ginde *et al.*, 2010). The International Osteoporosis Foundation has recently mapped 25(OH)-vitamin D concentrations across the globe using available data (Wahl *et al.*, 2012).

9.2.2 Assessment of normal range and measurement of 25(OH)-vitamin D

Circulating 25(OH)-vitamin D is the major transport form of vitamin D and is the most appropriate for measurement. There are several different methods available to measure 25(OH)-vitamin D. The gold standard is seen to be gas chromatography–mass spectrometry (GC–MS), but this technique is expensive and time-consuming. Many labs use commercial kits, which are usually radio-immunometric or chemi-luminescence assays. These tend to be less accurate than GC–MS and high-performance liquid chromatography (HPLC), and also discriminate less well between the D₂ and D₃ forms (Jones, 2006). 1,25(OH)₂-vitamin D is an adaptive hormone, maintaining the serum calcium concentration, and therefore its level will reflect prevailing conditions such as calcium intake, and defining a universal normal level may not be meaningful (Holick and Garabedian, 2006). The concept of what is the normal range for 25(OH)-vitamin D is highly controversial at the moment. Historically, serum levels have been classed as ‘replete’ (>50 nmol/l), insufficient (25–50 nmol/l) or deficient (<25 nmol/l). (Older studies often use ng/ml as the unit of measurement: 1 ng/ml = 2.5 nmol/l.)

The distinction between replete and insufficient has been made on the basis of whether there is a secondary rise in PTH, and deficient as a lower cut-off below this level. Other approaches have related serum 25(OH)-vitamin D concentrations to small intestine fractional calcium absorption or markers of bone turnover. A recent review of over 50 studies relating 25(OH)-vitamin D concentrations to those of PTH found that, where a threshold in 25(OH)-vitamin D concentration was observed (which was not in all), this ranged from 25–125 nmol (Sai *et al.*, 2011), clearly demonstrating that using this technique to define normality is extremely problematic. Similar issues are apparent with other such approaches. Furthermore, a study of bone biopsies in 675 cadavers found that that, although there was no evidence of mineralization defects with 25(OH)-vitamin D concentrations >75 nmol/l, even below 25 nmol/l a substantial number of biopsies appeared to be normally mineralized (Priemel *et al.*, 2010). Clearly it is difficult to extrapolate from a serum 25(OH)-vitamin D concentration to a pathological outcome in the absence of other clinical data; the evidence suggests that 25(OH)-vitamin D concentration should be treated purely as an aid to diagnosis rather than as a disease in itself (Harvey and Cooper, 2012).

Despite the push towards a higher threshold in the USA over the last decade, the Institute of Medicine report favoured a threshold of 50 nmol/l (IOM, 2010), with a recent European symposium adopting an even more conservative approach, concluding that the best evidence for harm was at levels below 25 nmol/l and that the current evidence base did not exclude an adverse effect of higher levels of

vitamin D (Lanham-New *et al.*, 2011). A further controversy is whether the threshold should differ by outcome, for example whether the level required for optimal bone function is the same as the level needed to prevent the potential increased risk of other diseases, such as colon cancer, which have been associated with vitamin D deficiency in observational studies.

9.3 Disease states associated with vitamin D

9.3.1 Skeletal

Historically, the main diseases associated with low vitamin D status have been those of the skeleton. Thus, rickets in infants and osteomalacia in adults are well-established sequelae of inadequate vitamin D stores (Lips *et al.*, 2009). In developing countries inadequate calcium or phosphorus intake may also lead to a similar histological picture of undermineralized protein matrix. The clinical presentation in both adults and children is one of bone and muscle pain, proximal muscle weakness and biochemical abnormalities including low serum calcium, low 25(OH)-vitamin D and raised PTH and alkaline phosphatase (Lips *et al.*, 2009). Specific skeletal abnormalities such as those at the costal margins of the ribs and splaying of the distal metastasis of the femur may be observed in infant rickets. Bone deformity, as a result of weakened bone structure may be present in both adults and children.

Bone biopsy is currently the only way to diagnose osteomalacia histologically, but the clinical and biochemical picture usually allows the diagnosis to be made. One recent study of 675 human subjects at autopsy has demonstrated that there is no threshold in circulating 25(OH)-vitamin D level below which osteomalacic changes on bone biopsy are always seen (Priemel *et al.*, 2010), but that no changes were seen in samples where the circulating concentration was above 75 nmol/l. Maternal vitamin D deficiency in pregnancy has been associated with neonatal hypocalcemia (Purvis *et al.*, 1973) and other adverse birth outcomes, such as craniotabes and widened growth plates, suggestive of rachitic (rickets-like) change (Reif *et al.*, 1988). Indeed a recent study demonstrated rachitic-like widening of the fetal distal femoral metaphysis relative to its length, scanned by ultrasound at 19 and 34 weeks, in fetuses of mothers with low levels of circulating 25(OH)-vitamin D, implying a relatively early effect (Mahon *et al.*, 2010). Infants of mothers with low vitamin D intake may have lower calcium levels at day four post-delivery (Paunier *et al.*, 1978), and be at greater risk of adverse post-natal bone development (Javaid *et al.*, 2006; Harvey *et al.*, 2008).

9.3.2 Other diseases

In recent years, evidence has accumulated from mainly observational studies that a low circulating level of 25(OH)-vitamin D may be a risk factor for a panoply of different diseases. Thus conditions ranging from colon cancer to type I diabetes and multiple sclerosis have all been associated with vitamin D deficiency (Holick,

2004a). Many cell types in the body express the VDR and may have the ability to locally convert 25(OH)-vitamin D to 1,25(OH)₂-vitamin D (Sharma, 2000; Holick, 2003, 2004b). There is thus a good biological basis to support the notion that vitamin D may be critical in a whole range of diseases. Studies in the laboratory have demonstrated that 1,25(OH)₂-vitamin D has biological actions in several different cell types. However, most clinical data in humans come from observational studies, many of which are cross-sectional, relating either dietary intake of vitamin D or circulating 25(OH)-vitamin D concentrations to disease outcomes, making issues of reverse causality and residual confounding critical. It is important to recognize whether a paper is reporting data on the main circulating form of vitamin D, i.e. 25(OH)-vitamin D, or on the active form 1,25(OH)₂-vitamin D. Often clinically-based epidemiological papers use the former and are really considering vitamin D as a supplement; in contrast laboratory-based studies mostly use the latter, effectively treating the active form of vitamin D as a pharmacologic agent. Currently there are many discrepancies in the evidence base, particularly between epidemiological and laboratory studies. Some of these issues may result from the observational nature of the clinical evidence and may be resolved with intervention studies and a greater number of longitudinal designs. However, other areas of disagreement may result from a fundamental difference between the effect of improving circulating 25(OH)-vitamin D and the use of the active 1,25(OH)₂-vitamin D as treatment.

9.4 Vitamin D and the immune system

The discovery of the VDR in cells of the immune system led to the initial interest in vitamin D as an immunoregulatory agent (Bhalla *et al.*, 1983; Provvedini *et al.*, 1983). Resting T cells express low levels of VDR, which are upregulated following activation (Veldman *et al.*, 2000; Mahon *et al.*, 2003). All immune cells that have been evaluated express the VDR and, in most cell types, activation induces further expression of the VDR. The mechanisms by which vitamin D regulates immune function have largely been investigated *in vitro* using 1,25(OH)₂-vitamin D addition to cells in culture and/or in experimental animals treated with 1,25(OH)₂-vitamin D or transgenic mice that lack the VDR. As a result some of the mechanisms noted may be due to the pharmacological effects of 1,25(OH)₂-vitamin D instead of the physiological effects of vitamin D.

9.4.1 Vitamin D as an immune regulator

The innate immune system is the body's first response following infection or injury. Innate immune cells such as macrophage and dendritic cells (DC) recognise pathogens via pattern-recognition receptors and become activated. These activated cells then become antigen-presenting cells (APC); they process proteins into peptides and present these peptides to T cells in the local environment. The effect of 1,25(OH)₂-vitamin D on the APC is to induce interleukin (IL)-10 and

suppress IL-12 which has indirect effects on the development of T cell subsets (Adorini and Penna, 2009). In addition, $1,25(\text{OH})_2$ -vitamin D has been shown to prevent DC maturation that also leads to suppression of the induction of T cells (Griffin *et al.*, 2001; Adorini and Penna, 2009). More recently, there has been considerable interest in the role of vitamin D as a regulator of innate antibacterial immune responses. In neutrophils, $1,25(\text{OH})_2$ -vitamin D suppressed the ability of lipopolysaccharide to induce IL-1 β expression as well as inhibiting some antimicrobial genes (Takahashi *et al.*, 2002). $1,25(\text{OH})_2$ -vitamin D and $25(\text{OH})$ -vitamin D have also been shown to induce antibacterial peptides in macrophages *in vitro* (Liu *et al.*, 2006; Liu and Modlin, 2008). In the presence of $1,25(\text{OH})_2$ -vitamin D, macrophages and DC produce less IL-12, more IL-10 and more antibacterial peptides.

The effect of $1,25(\text{OH})_2$ -vitamin D on CD4^+ T cells has been studied extensively. Several different investigators using several different models and systems have shown that type 1-mediated T helper (Th1) cell responses are reduced *in vitro* and *in vivo* in the presence of $1,25(\text{OH})_2$ -vitamin D (Lemire, 1992; Deluca and Cantorna, 2001; Mathieu and Adorini, 2002; Cantorna and Mahon, 2004; Mathieu *et al.*, 2004). Human T cell cultures show that $1,25(\text{OH})_2$ -vitamin D inhibits proliferation of T cells and production of IL-2 and interferon (IFN)- γ (Rigby *et al.*, 1984; Tsoukas *et al.*, 1989; Muller *et al.*, 1993). *In vitro* $1,25(\text{OH})_2$ -vitamin D suppresses cytokines produced by either the Th1 and/or the Th17 cells (Reichel *et al.*, 1987; Cippitelli and Santoni, 1998; Tang *et al.*, 2009). There are conflicting reports of effects in Th2 cells. Several groups have shown that $1,25(\text{OH})_2$ -vitamin D induces production of IL-4, while one group has shown that $1,25(\text{OH})_2$ -vitamin D suppresses IL-4 (Boonstra *et al.*, 2001; Pichler *et al.*, 2002; Imazeki *et al.*, 2006). A fourth subset of CD4^+ T cells, the regulatory (reg) T cell, has been shown to increase in number in the presence of $1,25(\text{OH})_2$ -vitamin D (Barrat *et al.*, 2002; Gregori *et al.*, 2002). Thus, the *in vitro* effects of $1,25(\text{OH})_2$ -vitamin D on Th cells are to inhibit Th1 and Th17 responses, and to induce Treg cells.

Conventional CD4^+ T cell responses in VDR knockout (KO) mice are not very different to those in the wild-type (WT). The numbers and types of CD4^+ T cells are the same in VDR KO and WT mice (Yu and Cantorna, 2008; Yu *et al.*, 2008). *In vitro*, analysis of the function of VDR KO CD4^+ T cells shows that they proliferate more rapidly in a mixed lymphocyte reaction and produce more IFN- γ than their WT counterparts (Froicu *et al.*, 2003). VDR KO Treg cells are present in normal numbers and function as well as their WT counterparts (Yu *et al.*, 2008). Conversely, a newly described population of T cells, the natural killer T (NKT) cells, absolutely require vitamin D for both development and function (Yu and Cantorna, 2008). Mature NKT cells can be CD4^+ or CD4^- . VDR KO mice have normal numbers of conventional T cells but express very few NKT cells, that in addition are functionally defective.

CD8^+ cytotoxic T cells have been shown to express more VDR than CD4^+ T cells (Veldman *et al.*, 2000). However, the inhibitory effects of $1,25(\text{OH})_2$ -vitamin D *in vivo* were shown to be intact in the absence of CD8^+ T cells (Meehan and DeLuca, 2002a). Vitamin D has been shown to be important in the regulation of

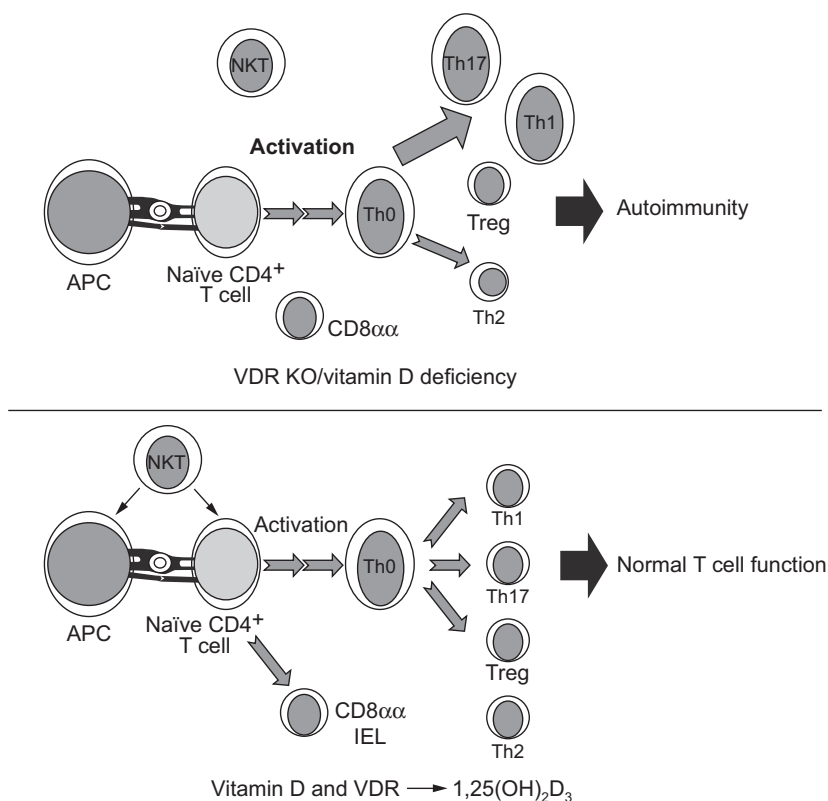


Fig. 9.3 The role of vitamin D in CD4⁺ T cell development and function. IEL, intestinal intraepithelial lymphocytes.

CD8αα T cells that reside in the gastrointestinal tract (Yu *et al.*, 2008). VDR KO mice have half as many CD8αα T cells in the gut than WT mice (Yu *et al.*, 2008). In addition, the CD8αα T cells in the VDR KO mice produce less IL-10 than those in the WT (Yu *et al.*, 2008). B cells have also been shown to be vitamin D targets (Lemire *et al.*, 1984, Bruce *et al.*, 2008). 1,25(OH)₂-vitamin D has been shown to inhibit B cell proliferation and IgG and IgM production *in vitro* (Lemire *et al.*, 1984). VDR KO mice over-produce IgE antibodies and as VDR KO mice age, they also over-express IgG antibodies (Bruce *et al.*, 2009). More work should be done to determine the effects of the VDR on B cells.

In summary, the effects of 1,25(OH)₂-vitamin D on APC include keeping DC in an immature state, reducing production of inflammatory cytokines such as IL-12 and inducing production of anti-inflammatory cytokines such as IL-10. In T cells 1,25(OH)₂-vitamin D reduces Th1 and Th17 responses while inducing Treg cells. Conversely, 1,25(OH)₂-vitamin D does not have a consistent effect on Th2 cells. In VDR deficiency, Th1 cells over-produce IFN-γ, and CD4⁺ T cells proliferate more. Expression of the VDR is required for normal development and function of both NKT cells and CD8αα T cells. In addition, VDR KO mice over-produce IgE

and IgG. Together, the effects of vitamin D on immune function are to control the development of Th1-mediated immune responses and to fine-tune and eventually shut off the Th1 cells (Fig. 9.3).

9.4.2 The immune system as an endocrine organ for vitamin D

There has been a great deal of attention given to the possibility that the immune system might be able to produce $1,25(\text{OH})_2$ -vitamin D for its own use. The data are convincing that under normal conditions the only organ to express the $1\text{-}\alpha$ -hydroxylase is the kidney (Gray *et al.*, 1979; Reeve *et al.*, 1983; Shultz *et al.*, 1983; Vanhooke *et al.*, 2006). Evidence that uses mRNA analysis of the $1\text{-}\alpha$ -hydroxylase and/or poorly characterised antibodies to the $1\text{-}\alpha$ -hydroxylase cannot substitute for demonstration of enzyme activity (Abreu *et al.*, 2004; Kallay *et al.*, 2005; Hewison *et al.*, 2007; Liu *et al.*, 2008). There is evidence that during disease the $1\text{-}\alpha$ -hydroxylase may be expressed in the immune system (Barbour *et al.*, 1981; Bosch, 1998; Abreu *et al.*, 2004; Kavathia *et al.*, 2010). In addition, human monocyte-derived macrophages have been shown to produce $1,25(\text{OH})_2$ -vitamin D *in vitro* (Hewison *et al.*, 2003). Macrophages from a patient with sarcoidosis, but not from other patients with other lung diseases, showed $1,25(\text{OH})_2$ -vitamin D production (Barbour *et al.*, 1981). Human macrophages cultured *in vitro* with *Mycobacteria tuberculosis* were shown to produce $1,25(\text{OH})_2$ -vitamin D from the $25(\text{OH})$ -vitamin D in the cultures (Liu *et al.*, 2006).

The presence of $1\text{-}\alpha$ -hydroxylase in DC and macrophages suggests the possibility that locally-produced $1,25(\text{OH})_2$ -vitamin D has regulatory autocrine and paracrine properties at the site of inflammation. This is an attractive hypothesis that suggests that locally controlled and produced $1,25(\text{OH})_2$ -vitamin D might regulate immune function without causing systemic effects. However, at present there are no good examples demonstrating that immune-derived $1,25(\text{OH})_2$ -vitamin D occurs *in vivo* in healthy people and/or animals. The best examples of immune-derived $1,25(\text{OH})_2$ -vitamin D are in very sick patients. Of interest is that with the resolution of the disease there is a concomitant normalisation of the $1,25(\text{OH})_2$ -vitamin D and calcium levels (Bell *et al.*, 1979; Bosch, 1998; Kavathia *et al.*, 2010). These observations raise the possibility that immune-produced $1,25(\text{OH})_2$ -vitamin D may contribute to disease as well as being a mechanism to locally control immune function.

9.4.3 Vitamin D and autoimmunity

Based on the mechanisms by which $1,25(\text{OH})_2$ -vitamin D and expression of the VDR regulate immune function, it would seem likely that additional vitamin D might be used to suppress diseases where Th1-mediated immune responses are pathogenic. Multiple sclerosis (MS), type-1 diabetes, arthritis and inflammatory bowel disease (IBD) are among the Th1-mediated autoimmune diseases linked to the over-production of IFN- γ . Several animal models of Th1-mediated autoimmunity have been shown to be suppressed following treatment with $1,25(\text{OH})_2$ -vitamin D

(Cantorna *et al.*, 1998a, 1996; Cantorna *et al.*, 2000; Zella and DeLuca, 2003; Zella *et al.*, 2003). Furthermore, vitamin D deficiency has been shown to exacerbate IBD and diabetes in mice (Cantorna *et al.*, 2000; Zella *et al.*, 2003). Experiments that looked at the mechanisms of $1,25(\text{OH})_2$ -vitamin D-mediated autoimmune suppression are consistent with inhibition of Th1 and Th17 responses and upregulation of FoxP3 regulatory T cells (Cantorna *et al.*, 1998c; Barrat *et al.*, 2002; Gregori *et al.*, 2002; Daniel *et al.*, 2008). The data clearly support a beneficial role for $1,25(\text{OH})_2$ -vitamin D in experimental Th1-mediated autoimmunity.

There is reason to believe that perhaps the data in experimental autoimmunity might translate to human disease. Vitamin D insufficiency has been correlated to several autoimmune diseases including MS, arthritis and IBD (Merlino *et al.*, 2004; Munger *et al.*, 2006; Katz, 2006; Pappa *et al.*, 2006). The hypothesis has been put forward that vitamin D might be an environmental factor that affects the risk of developing type 1-mediated autoimmunity in genetically susceptible individuals. Most of the data are either observational or retrospective although there have been a small number of studies that have shown that vitamin D supplementation has beneficial effects on IBD and MS among others (Burton *et al.* 2010; Jorgensen *et al.*, 2010; Kriegel *et al.*, 2011). Pharmacological experiments using either $1,25(\text{OH})_2$ -vitamin D or analogues of $1,25(\text{OH})_2$ -vitamin D are still in the early safety phases and therefore efficacy has not yet been shown (Kriegel *et al.*, 2011). In addition, a number of polymorphisms in the VDR and several of the metabolic and catabolic vitamin D enzymes have been linked to autoimmunity (Simmons *et al.*, 2000; Dresner-Pollak *et al.*, 2004; Mory *et al.*, 2009). This emphasises a need for additional experimentation to determine whether people at risk for autoimmune disease might have different vitamin D requirements compared to those that are not at risk for autoimmune disease. Experiments have not yet been done to prove the beneficial effects of vitamin D supplementation for any autoimmune disease in humans. However, together with the experimental data from animal models and *in vitro* assays, association data in humans and small interventions do provide some support for increasing vitamin D intakes in people at risk for autoimmunity (family members of autoimmune patients).

9.4.4 Vitamin D and infection

There has been a great deal of recent interest in the possibility that vitamin D might be useful for treating infectious diseases (reviewed in Bruce *et al.*, 2010). Based on the fact that Th1 responses are suppressed by $1,25(\text{OH})_2$ -vitamin D, it would seem possible that infections that require Th1 responses for clearance might be made worse by vitamin D (Cantorna *et al.*, 2008). In fact, there are data showing that $1,25(\text{OH})_2$ -vitamin D treatment of macrophages reduced their ability to kill the intracellular organism *Listeria monocytogenes* (Helming *et al.*, 2005). Additional evidence showed that $1,25(\text{OH})_2$ -vitamin D had neither a beneficial nor a detrimental effect on the ability of mice to clear infections with two different organisms that require Th1-mediated immunity for clearance (Cantorna *et al.*, 1998b, Hullett *et al.*, 1998). However, more recently it has been shown that macrophages produce

1,25(OH)₂-vitamin D that induces antimycobacterial peptides *in vitro* (Liu *et al.*, 2006). This presents a conundrum since the data show that strong Th1-mediated immune responses are required for clearance of *M. tuberculosis* from infected macrophages. At present, there is a paucity of data that fail to provide any type of convincing picture of what the effect of vitamin D, 1,25(OH)₂-vitamin D and/or vitamin D deficiency would be on the ability of a host to fight an infection (Bruce *et al.*, 2010).

9.4.5 Vitamin D and asthma

Allergic asthma results from a type 1 hypersensitivity reaction that is characterised by the generation of Th2 cells that drive the production of antibodies of the IgE isotype. Experimentally, the effect of 1,25(OH)₂-vitamin D on Th2 responses seems to depend on how the experiment was carried out since there are examples of increases, decreases and no effects reported (Boonstra *et al.*, 2001; Pichler *et al.*, 2002; Imazeki *et al.*, 2006). In animal models of allergic asthma the same picture emerges; some symptoms have been shown to increase, some have been shown to decrease and others report no change in the symptoms associated with 1,25(OH)₂-vitamin D treatments (Matheu *et al.*, 2003; Topilski *et al.*, 2004; Wittke *et al.*, 2004). Similarly, vitamin D deficiency had no effect on development of allergic asthma while VDR KO mice were completely resistant to the development of allergic asthma (Wittke *et al.*, 2004). Evidence in humans is also somewhat mixed. Data from older small studies show that the incidence of wheeze is higher in children who received supplemental vitamin D (Wjst and Dold, 1999). More recently it has been reported that vitamin D deficiency was associated with an increased incidence of asthma (Sutherland *et al.*, 2010). At present, it is unclear what the role of vitamin D might be in the development of asthma.

9.4.6 Tissue-specific effects of vitamin D on immune function

The potential tissue effects of vitamin D can be seen when comparing the effects of vitamin D on experimental autoimmunity in the central nervous system (CNS; MS) versus the gastrointestinal (IBD) tract. The immune responses that cause pathology in IBD and MS are similar: both IBD and MS are Th1 cell-mediated autoimmune diseases although the targets of the immune mediated attack are different. Vitamin D deficient IL-10 KO mice develop a fulminating form of IBD that is lethal in 50% of the animals by five weeks of age (Cantorna *et al.*, 2000). VDR and IL-10 double KO mice develop the disease more quickly than the vitamin D-deficient IL-10 KO and it results in the lethality of all double KO mice in three weeks (Froicu *et al.*, 2003). Vitamin D-sufficient IL-10 KO mice begin to show symptoms of IBD at nine weeks and most of the mice never develop a fulminating form of the disease (Cantorna *et al.*, 2000). Other animal models of IBD (dextran sodium sulfate-induced) are also more severe in VDR KO mice (Froicu and Cantorna, 2007). Vitamin D deficiency accelerates the development of experimental autoimmune encephalitis (EAE) (murine MS) but only in female mice and only

in one particular strain of mice (Cantorna *et al.*, 1996; Fernandes de Abreu *et al.*, 2010; Wang *et al.*, 2012). Similarly, VDR KO mice are less susceptible to EAE than WT mice (Meehan and DeLuca, 2002b; Wang *et al.*, 2012). There are potentially multiple explanations for these differences. However, it is known that the epithelial cells of the gastrointestinal tract express the VDR and the gastrointestinal tract is a critical target of vitamin D. Therefore the vitamin D regulation of inflammation in the gut will be both within and outside of the immune system while the effects of vitamin D in the CNS may be restricted to regulation of the immune system only.

9.5 Predictions and state of the evidence to support vitamin D interventions to alter immune function

Currently there is compelling experimental evidence to make the case that vitamin D status, 1,25(OH)₂-vitamin D and the VDR are important inhibitors of autoimmunity mediated by Th1 and Th17 cells. Experimental data on the effects of vitamin D on other types of immune responses including Th2-mediated immune responses and/or anti-infectious immunity are sometimes contradictory and paradoxical. However, even in autoimmune disease there are so far no compelling data that shows that vitamin D supplementation in humans is efficacious. Even if vitamin D would be effective in autoimmune disease, it is unclear what dose, how often and/or what the target 25(OH)-vitamin D levels in the blood should be, particularly as the relationship between concentrations of 25(OH)-vitamin D and 1,25(OH)₂-vitamin D is not readily predictable. At present, there is no compelling evidence to support taking additional vitamin D for asthma, or to help prevent or treat any infectious disease.

9.6 Conclusion

Significant progress has been made in understanding the mechanisms by which vitamin D regulates immune function. In particular, experimental evidence has shown that 1,25(OH)₂-vitamin D and the VDR regulate Th1- and Th17-mediated autoimmunity. What is less clear is whether increasing vitamin D intakes might also regulate Th1- and Th17-mediated immunity and be beneficial for treating autoimmune disease. In addition, the evidence does suggest that some immune cells (NKT cells and CD8αα T cells) do require adequate vitamin D status and VDR signalling to develop and function normally. Additional experimentation is required to understand what other immune responses and tissues are regulated by vitamin D, 25(OH)-vitamin D and 1,25(OH)₂-vitamin D. These mechanistic experiments are useful for identifying populations that might benefit from vitamin D supplementation. While it is possible that improving 25(OH)-vitamin D status in the population may have a beneficial effect on immune function, the current evidence base is insufficient to make a definite recommendation, even in those with existing immune disease.

9.7 References

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Part III

Other dietary constituents, immunity and inflammation

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10

Intestinal bacteria and probiotics: effects on the immune system and impacts on human health

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Abstract: The intestinal microbiota and some probiotics are known to interact with the host's immune system, thereby influencing both health status and disease risk. These interactions contribute to immune response activation against dangerous stimuli, as well as to the activation of regulatory mechanisms that avoid uncontrolled inflammation and chronic pathologies. To date, most of the applications of probiotics with immunomodulatory traits have been focused on prevention and treatment of different clinical conditions, including infections, chronic inflammatory bowel disorders and allergies. Herein, the modes of action of intestinal bacteria and probiotics on the immune system and their uses in human health are revised.

Key words: intestinal bacteria, probiotics, immune system, lymphocyte differentiation, T cells.

10.1 Introduction

The mucosa of the intestinal tract is one of the largest surfaces of the human body that is exposed to environmental antigens and to a large number of commensal bacteria (microbiota). To peacefully co-exist with this antigenic pressure, the epithelium and the mucosal immune system coordinate a network of immunological responses that provide both protection against pathogens and tolerance to innocuous antigens and bacteria. The intestinal epithelium consti-

tutes a physical barrier that excludes the entry of most antigens prior to immune activation, and also participates in the regulation of immune homeostasis. The primary defensive mechanisms include the production of mucus and other secretions, regulation of paracellular permeability and synthesis of antimicrobial peptides by Paneth cells. Moreover, the epithelium and gut-associated lymphoid tissue (GALT) develop complex interactions and immune responses to different stimuli, which involve the production of cytokines, chemokines and activation of different effector T cells. Altogether this ensures pathogen elimination and avoids over-reactions that can lead to tissue damage and pathologic inflammatory conditions.

The commensal microbiota is also known to be involved in the post-natal development and optimal functioning of the immune system, influencing both innate and acquired immunity. Commensal bacteria can enhance the intestinal epithelial barrier function by modulating mucin gene expression and glycosylation by goblet cells, which influence bacterial adhesion and invasion (Freitas *et al.*, 2005; Caballero-Franco *et al.*, 2007). Some commensal bacteria also induce the secretion of antimicrobial peptides (defensins and angiogenins) by intestinal Paneth cells (Hooper *et al.*, 2003; Vaishnava *et al.*, 2008) and regulate intestinal permeability (Lutgendorff *et al.*, 2008). The intestinal microbiota also has an enormous impact on the development of mucosal and systemic immunity, as evidenced by studies conducted with germ-free and colonized mice. In germ-free mice, the GALT is immature, with a low content of lamina propria T cells, immunoglobulin (Ig) A-producing B cells and intraepithelial T cells, and the serum immunoglobulin levels are reduced and spleens are smaller. These deficiencies are, however, restored after colonization with commensal bacteria (Tlaskalová-Hogenová *et al.*, 2004). Human studies also report that the bacteria colonizing the newborn intestine influences production of salivary secretory IgA, numbers of circulating IgA and IgM antibody-producing cells and expression of innate immune receptors (e.g. Toll-like receptors, TLRs) in peripheral blood monocytes (Grönlund *et al.*, 2000; Rautava *et al.*, 2006). Prospective observational studies also report that alterations in the microbiota composition early in life precede the development of atopic dermatitis (Kopp and Selfeld, 2009), suggesting the microbiota plays a role in the risk of developing immune-mediated diseases.

Evidence on the influence exerted by the intestinal microbiota on human physiology has led to increased interest in the selection of specific bacterial strains with health promoting properties, generally known as probiotics (FAO/WHO, 2002). The probiotics used for human consumption are mainly strains belonging to the genera *Bifidobacterium* and *Lactobacillus*. To date, most of the applications of probiotics with immunomodulatory traits have been focused on prevention and treatment of different clinical conditions, including infections, chronic inflammatory bowel disorders (IBDs) and allergies. Herein, the modes of action of intestinal bacteria and probiotics on the immune system and their uses in human health are revised.

10.2 Mechanisms of action of intestinal bacteria and probiotics on the immune system

10.2.1 Recognition of microbial signals by epithelial and innate immune cells

It is now accepted that the colonization of the intestinal tract by microbiota triggers a response in the mucosal immune system that involves recognition of microbial signals by innate receptors called pattern-recognition receptors (PRRs), modulating the mucosal immune cell function. These receptors are expressed by epithelial cells and antigen-presenting cells (DCs and macrophages) and are responsible for the initial recognition of specific microbial components (e.g. RNA, DNA motifs, cell-wall components, etc.), the discrimination between pathogens and harmless microbes and the development of appropriate innate and acquired immune responses (Michelsen and Arditi, 2007). These receptors include the TLR family, specific for lipid molecular patterns (TLR 1, 2, 4 and 6) and bacterial proteins (TLR5) expressed on the plasma membrane (West *et al.*, 2006) and for viral RNA or DNA receptors localized in the endosomal compartment (TLR 3, 7, 8 and 9). Other relevant receptors are Nod-like receptors (NLRs) soluble in cytosol and the transmembrane G protein-coupled receptors (GPCRs) expressed in haematopoietic and non-haematopoietic cells of the intestine (Hill and Artis, 2009). Table 10.1 summarizes the ligands recognized by these innate immune receptors.

Upon ligand-binding, TLRs promote signal divergence via interactions with different adaptor proteins, thereby activating three major signalling pathways: nuclear factor kappa B (NF- κ B), the mitogen-activated protein kinases (MAPKs) and interferon (IFN) regulatory factors (IRFs) (Lee and Kim, 2007). This leads to the expression of inflammatory genes, encoding cytokines, cytokine receptors, immunoregulatory proteins, adhesion molecules, stress-associated proteins and other mediators as well as the recruitment of other immune cells (T cells, basophils, neutrophils, dendritic cells and natural killer cells). Altogether, these activate an inflammatory response that can lead to pathogen clearance.

Several studies report that the influence of bacteria on TLR expression differs between commensal and pathogenic bacteria, as well as between Gram-positive and Gram-negative bacteria, which partly explains their different immune effects. In mice, Gram-positive bacteria (*L. acidophilus* CRL 924, *L. delbrueckii* subsp. *bulgaricus* CRL 423 and *L. casei* CRL 431) increased the number of TLR2-positive cells, mainly via interaction with lipoteichoic acids (Grangette *et al.*, 2005), while Gram-negative bacteria (*Escherichia coli* 129 and *E. coli* 13-7) increased that of TLR4-positive cells parallel to different cytokine induction (interleukin (IL)-10 versus IL-12) (Dogi *et al.*, 2008). In wild-type mice, but not in TLR2 or TLR4 knockout mice, the probiotic strain *E. coli* Nissle, 1917 ameliorated colitis and decreased pro-inflammatory cytokine secretion, suggesting that the effects were mediated via TLR2- and TLR4-dependent pathways (Grabig *et al.*, 2006). The immune effects of the intestinal microbiota and some probiotics are also mediated by regulating the activation of different components of the TLR signalling pathways (NF κ B/Rel, MAPK and caspase-dependent signalling

Table 10.1 Human pattern-recognition receptors and their ligands

Receptor	Ligand	References
TLR1/TLR2	Lipoproteins/lipopeptides, lipoarabinomannan, and peptidoglycans from Gram-positive and Gram-negative bacteria and mycoplasma, lipoteichoic acid	Takeuchi <i>et al.</i> , 1999; Schwandner <i>et al.</i> , 1999; Takeda <i>et al.</i> , 2003
TLR2	Pam3Cys-Ser-(Lys)4, atypical LPS from nonenterobacteria	Takeuchi <i>et al.</i> , 2002; Akira <i>et al.</i> , 2006
TLR2/6	Diacyl lipopeptide	Hoebe <i>et al.</i> , 2005
TLR3	dsRNA (viral), poly (I : C)	Sen and Sakar, 2005; Alexopoulou <i>et al.</i> , 2001
TLR4	LPS, lipoteichoic acid, mannuronic acid polymers (bacteria), heat-shock protein 60, heat-shock protein 70	Gazzinelli <i>et al.</i> , 2004; Nasu and Narahara, 2010
TLR5	Flagellin (bacteria)	Hayashi <i>et al.</i> , 2001
TLR7, TLR8	ssRNA	Heil <i>et al.</i> , 2004
TLR9	Unmethylated CpG DNA (bacteria, protozoa, virus)	Lee <i>et al.</i> , 2003; Tabeta <i>et al.</i> , 2004
NOD1	γ -D-glutamyl- <i>meso</i> -diaminopimelic acid	Girardin <i>et al.</i> , 2003
NOD2	Muramyl dipeptide	Girardin <i>et al.</i> , 2003
GPR109A	Butyrate	Thangaraju <i>et al.</i> , 2009

cascade), thereby inducing different transcriptional responses and different patterns of cytokine production. For example, in HT-29 cells subjected to pro-inflammatory stimuli, DNA from the probiotic mixture VSL3 inhibited IL-8 secretion, reduced p38 MAPK expression, delayed NF κ B activation, stabilized levels of I κ B and inhibited proteasome function (Jijon *et al.*, 2004). Also, *L. reuteri* ATCC PTA 6475 was shown to suppress tumour necrosis factor (TNF) production by lipopolysaccharide (LPS)-activated monocytes and primary monocyte-derived macrophages from children with Crohn's disease by inhibiting the activation of MAPK-regulated c-Jun and the transcription factor AP-1 (Lin *et al.*, 2008).

NOD1 and NOD2 are the best-characterized NLRs in intestinal cells and play a role in detecting intracellular micro-organisms. NOD1 is expressed in all cell types, including intestinal cells, and required for NF κ B activation by Gram-negative bacterial infection, once the bacteria have bypassed TLR activation (Fukazawa *et al.*, 2008). NOD2 expression is induced in small intestinal epithelial cells and NOD2 mutations are associated with defective IL-10 production and Crohn's disease (Netea *et al.*, 2004). The role of the GPCRs receptors is less well known, but it is now recognized that metabolites produced by the intestinal microbiota (e.g. acetic, butyric and propionic) could play a role in their activation (Pryde *et al.*, 2002). For example, in intestinal epithelial cells the receptor GPR109A is activated by butyric acid. This leads to the suppression of the NF κ B signalling pathway (Kumar *et al.*, 2009; Thangaraju *et al.*, 2009) and to the reduction of pro-inflammatory cytokine production (TNF- α , TNF- β , IL-6 and IL-1 β) by lamina propria lymphocytes (LPLs) in Crohn's and ulcerative colitis patients (Segain *et al.*, 2000; Lührs *et al.*, 2002), involving the regulation of multiple cell populations.

10.2.2 Influence of intestinal and probiotic bacteria on lymphocyte differentiation

Signalling through TLR also stimulates the maturation of DCs, inducing their antigen presentation ability, switching on the chemokine receptor program and allowing cells to migrate into draining mesenteric lymph nodes (MLN), where they present antigens to naïve T and B cells. T-cell differentiation into Th1, Th2, Th17 or regulatory T cells depends on the TLRs involved and the identity of the cytokine interacting with naïve T cells, inducing a characteristic array of cytokine production during differentiation (Fig. 10.1). At the transcriptional level, Th1 is accompanied by T-bet and STAT4 activation, while Th2 is characterized by GATA3 and STAT5 transcription. Th1-biased responses are associated with clearance of intracellular pathogens and with some chronic inflammatory bowel diseases such as Crohn's disease. Th2-biased responses are related to clearance of helminth parasites and IgE-mediated allergic reactions. Some TLR-activated DCs are conditioned to induce differentiation of naïve CD4⁺ T cells into Th1 cells, providing high levels of the Th1-polarizing cytokine IL-12, whereas TLR5- and TLR2-activated DCs may promote the differentiation of Th2 cells or regulatory

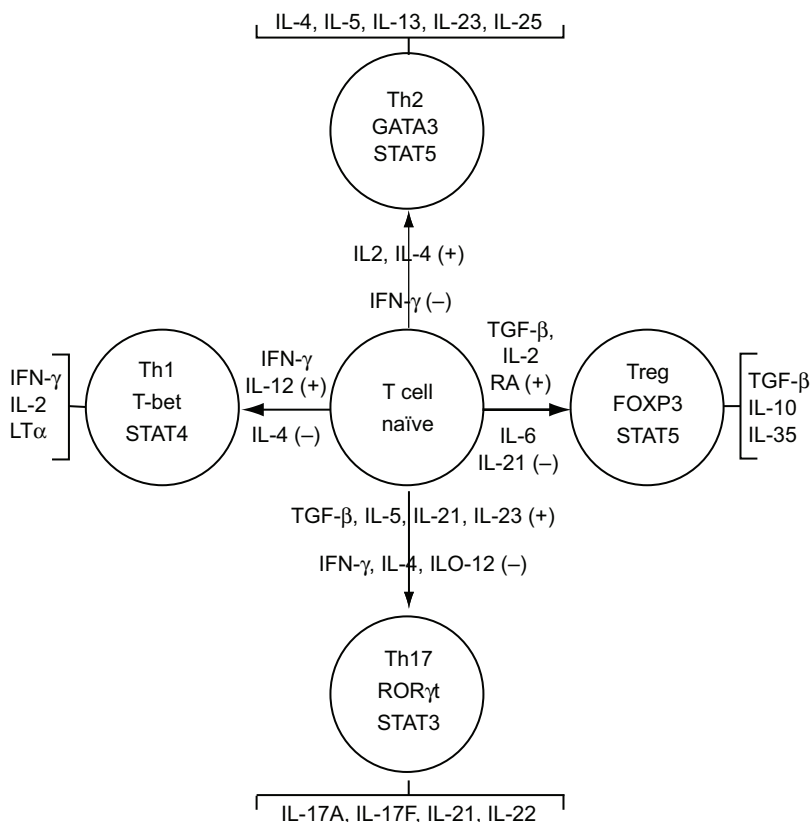


Fig. 10.1 CD4⁺ T cell subsets. Differentiation of naïve CD4⁺ T cells is stimulated positively by certain cytokines (+), and negatively regulated by others (-). Each T cell subset activates specific transcription factors producing a panel of cytokine characteristic to the subset.

T cells by producing high levels of anti-inflammatory cytokine IL-10 and low levels of IL-2 (Didierlaurent *et al.*, 2004; Lee and Kim, 2007).

Th17 represents the third member of effector T cells, characterized by production of cytokines such as IL-17, IL-17F, IL-6 and TNF (Langrish *et al.*, 2005) by ROR γ t and STAT3 activation. Differentiation of Th naïve on Th17 is mediated by IL-23 (Aggarwal *et al.*, 2003) or the combination of the pro-inflammatory cytokine IL-6 and transforming growth factor (TGF)- β . Th17 is associated with the production of inflammatory mediators, principally in autoimmune diseases. TLR2 has been shown to be relevant in Th17 polarization and in pathogenesis of autoimmune diseases.

Finally, antigen-specific regulatory T cells include different subtypes of CD4⁺ T cells: T regulatory 1 (Tr1) cells, which secrete high levels of IL-10, no IL-4 and no or low levels of IFN- γ ; and T helper 3 (Th3) cells, which secrete high levels of TGF- β (Mills, 2004), mediated by transcription of Foxp3 and STAT5. In the

absence of inflammation, a balance between effector and regulatory lymphocyte subpopulations is maintained through a tightly controlled cytokine network. Both IL-10 and TGF- β are important cytokines in directing naïve T-cell maturation to the generation of regulatory T cells (Treg). Treg include naturally thymus-produced CD4⁺ CD25⁺ Foxp3⁺ Treg cells and inducible type 1 IL-10-secreting cells (Akdis and Akdis, 2009a). Cells such as CD8⁺, $\gamma\delta$ T cells, DC, IL-10-producing B cells, natural killer (NK) cells and resident tissue cells can induce Treg differentiation, contributing to suppressive and regulatory events. Treg cells enable a systemic tolerance state to be achieved (Jutel *et al.*, 2003) by suppressing inflammatory properties of DC and their ability to induce effectors Th1, Th2 and Th17 cells, by promoting a tolerogenic DC phenotype development (Wing *et al.*, 2008) or by acting directly on eosinophils, mast cells, basophils and resident cells (Gri *et al.*, 2008; Nonaka *et al.*, 2008) and reducing cell migration to tissues (Ring *et al.*, 2006). In addition, Treg cells act directly under B cells, reducing allergen-specific IgE and inducing IgG4 (Meiler *et al.*, 2008).

In comparison with pathogenic bacteria, probiotic and commensal bacteria induce different cytokine production by stimulation of DCs, which may explain their different effects on lymphocyte differentiation. For example, O'Mahony *et al.* (2006) reported that commensal bacteria (e.g. *Lactobacillus* and *Bifidobacterium*) induced regulatory cytokine production (IL-10) by MLN and MLN-derived DCs, whereas pathogenic bacteria (*Salmonella*) induced Th1-polarizing cytokines (IL-12 and TNF- α). IL-10 production and CD83 expression were also induced by strains of the species *B. bifidum*, *B. longum* and *B. pseudocatenulatum* in cord blood DCs, polarizing the immune response toward a Th2 profile (Young *et al.*, 2004). *B. breve* BbC50_{SN} supernatant also induced DC maturation by up-regulating the expression of CD83, as well as CD86 and human leukocyte antigen (HLA) DR- and IL-10 production, which involved TLR2 interactions (Hoarau *et al.*, 2006). Probiotic strains of the product VSL#3, which include lactobacilli, streptococci and bifidobacteria, were also shown to influence DCs maturation. Individual strains displayed distinct immunomodulatory effects on DCs, and the most marked anti-inflammatory effects were exerted by bifidobacteria, which upregulated IL-10 production by DCs, decreased expression of the co-stimulatory molecules CD80 and CD40 and decreased IFN- γ production by T cells (Hart *et al.*, 2004). In addition, different strains of the same species may polarize immune responses in different directions via cytokine production regulation (Medina *et al.*, 2007; Ménard *et al.*, 2008). While some *B. longum* strains induced a Th2 orientation with high levels of IL-4 and IL-10, both secreted by splenocytes, and of TGF- β gene expression in the ileum of germ-free mice inoculated with bifidobacteria, other strains induced Th1 orientations with high levels of IFN- γ and TNF- α splenocyte secretions (Ménard *et al.*, 2008).

The probiotic mixture IRT5, including strains of the species *L. acidophilus*, *L. casei*, *L. reuteri*, *B. bifidum* and *Streptococcus thermophilus*, induced a regulatory phenotype in DC from MLN of mice. Conditioned DCs produce high levels of IL-10, TGF- β , COX-2 and indoleamine 2,3-dioxygenase (Ho-Keun *et al.*, 2009). The

suppression of NF κ B activation by prolonged JNK activation (induction of regulatory T cells by commensal and some probiotic bacteria may contribute to suppressing immune system activation and, thereby, to maintaining immune homeostasis and tolerance to self and harmless exogenous antigens (Hrncir *et al.*, 2008). In Balb/c mice, IRT5 upregulated Treg and downregulated T helper Th1, Th2 and Th17 cytokines in CD4⁺ cells (Ho-Keun *et al.*, 2009).

Strains of *L. reuteri* and *L. casei*, but not of *L. plantarum*, were shown to prime human monocyte-derived DCs to trigger regulatory T-cell development (Smits *et al.*, 2005). These regulatory T cells produced increased levels of IL-10 and were able to inhibit the proliferation of bystander T cells in an IL-10-dependent fashion. The binding of both *L. reuteri* and *L. casei*, the C-type lectin which is a DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), was necessary to induce regulatory T cells (Smits *et al.*, 2005).

B. infantis 35624 also induced regulatory T cells in mice, contributing to protecting the host against aberrant activation of the innate immune system in response to infection with *Salmonella enterica* serovar Typhimurium or to injection with LPS (O'Mahony *et al.*, 2008). In *B. infantis*-fed mice, a profound inhibition of infection and LPS-induced NF κ B activity, which preceded a reduction in *S. enterica* serovar Typhimurium numbers, and murine sickness behaviour scores, was detected. In addition, pro-inflammatory cytokine secretion, T-cell proliferation and DC co-stimulatory molecule expression were significantly reduced. In contrast, CD4⁺CD25⁺Foxp3⁺ T cell numbers increased significantly in the mucosa and spleen of mice fed *B. infantis*. In addition, adoptive transfer of CD4⁺CD25⁺ T cells transferred the NF κ B inhibitory capability of these Treg cells. Transfer of probiotic-treated DCs to mice also protected against 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, partly via induction of CD4⁺CD25⁺ regulatory cells (Foligne *et al.*, 2007a). The preventive effect of probiotic-pulsed DCs required MyD88, PRRs (TLR2- and NOD2)-dependent signalling and the induction of CD4⁺CD25⁺ regulatory cells in an IL-10-independent fashion. These effects were induced by *L. salivarius* Ls33 and *L. rhamnosus* Lr32 but not by *L. acidophilus* NCFM. In contrast, the capsular polysaccharide of the Gram-negative bacteria *Bacteroides fragilis* proved to be taken up by CD11c⁺ DC, leading to the production of IL-12 and increased Th1 response (Mazmanian *et al.*, 2005).

10.3 Evidence of the efficacy of probiotics in animal models

The intestinal microbiota is thought to play an important role in optimal functioning of the immune system, contributing to its activation to protect the host against pathogens and foreign antigens, but avoiding over-reactions. Therefore, the intervention in the intestinal ecosystem with probiotics has been evaluated as an alternative to modulate immune function and disease. Tolerance represents a state in which the immune response is not triggered against the host's own or exogenous innocuous antigens. However, alterations in reactivity could lead to the development of hypersensitivity and autoimmune diseases. Excessive reactivity against

harmless environmental antigens induces a state of hypersensitivity known as 'allergy', while excessive reactivity against the host's own antigens causes autoimmunity. In the following sections, evidence is reviewed of the potential role of some probiotic strains in different immune-mediated disorders gathered from preclinical and clinical studies

10.3.1 Infections

The use of probiotic micro-organisms in the prevention and treatment of infections has been one of the primary applications investigated in the field. Many examples illustrate the effects of potentially probiotic bacteria and their modes of action in animal models. These include modification of the environmental conditions and production of antimicrobial compounds (organic acids and antimicrobial peptides), competition for nutrients and adhesion sites, and modulation of the host immune defence mechanisms (Sanz *et al.*, 2007). For example, in a fatal animal model of Shiga toxin-producing *E. coli* O157:H7 infection, a *B. breve* strain produced high concentrations of acetic acid, consequently lowering the luminal pH, and this pH reduction was associated with increased animal survival (Asahara *et al.*, 2004).

Another example is the case of *L. salivarius* UCC118, which produces a bacteriocin called Abp118, involved in protection against *Listeria monocytogenes* infection in a mouse model (Corr *et al.*, 2007). Moreover, some strains are thought to enhance resistance to microbial pathogens by stimulating innate and acquired immune responses. These effects have been reported to be mediated through activating non-specific cell phagocytosis, increasing cytokine levels, increasing NK cell activity and/or increasing levels of immunoglobulins (Macfarlane and Cummings, 2002). For example, the administration of *B. lactis* NH019 to *S. typhimurium*-infected mice resulted in higher production of *S. typhimurium* pathogen-specific antibodies in serum and intestinal mucosa, as well as in increased phagocytic activity of peritoneal macrophages and blood borne neutrophils (Shu *et al.*, 2000). Strains of *B. bifidum* and *B. infantis* administered to rotavirus-infected mouse pups also increased virus-specific IgA levels in serum and the gastrointestinal tract (Qiao *et al.*, 2002). In addition, probiotics can exert protective effects in models of infection through the induction of Treg and regulatory DCs, preventing excessive inflammation. For example, the induction of Treg expression by *B. infantis* 35624 has been shown to mediate protection against excessive NF κ B activation via TLR4 triggered by *S. enterica* infection (O'Mahony *et al.*, 2008).

10.3.2 Allergy

In a hypersensitivity reaction, host exposure to allergens induces Th naïve cell differentiation into Th2 cells and B-cell class-switch into ϵ -immunoglobulin heavy-chain and IgE-specific allergen. IgE binds to Fc ϵ RI on mast cells and basophils and memory T and B cells are produced. In a second step, allergen re-exposure activates mast cells or basophils triggering the effector phase (Abbas and Lichtman, 2003). Continuous allergen exposure induces Th2 cell activation

characterized by cytokine IL-4, IL-5, IL-9 and IL-13 production, high levels of allergen-specific IgE production, eosinophilia, recruitment of inflammatory cells to inflamed tissues, mucus production and decreased threshold of smooth muscle contraction (Akdis *et al.*, 2009a,b). Studies in healthy individuals and several clinical trials have demonstrated that allergen-specific immunotherapy (allergen-SIT) represents the mean curative treatment of allergies, where antigen-specific T cells with immunoregulatory activity (Treg) are induced. On the other hand, reduction in Treg cell activity induces immune deregulation, a relevant factor in allergic disease development (Romagnani, 2004; Akdis *et al.*, 2009a). For example, Leung *et al.* (2004) found that Treg are decreased in atopic dermatitis (AD), supporting the theory that the increased suppressor activity of Treg could be a strategy to reduce damage caused by allergic disease.

From this perspective, several animal models have been employed to evaluate the role that probiotics could play in allergic diseases. For example, oral administration of *B. longum* AH1206 increased the number of Treg cells in lymphoid tissue and upregulated acid retinoic gene expression (Lyons *et al.*, 2010). It was also demonstrated that this strain was able to reduce levels of inflammatory cytokine TNF- α and IL-6 produced by bronchoalveolar cells and to decrease eosinophil recruitment in an ovalbumin (OVA) respiratory allergy mouse model. In an alimentary allergy model of OVA, *B. longum* AH1206 administration prevented the increase of OVA-specific IgE in serum. The results suggest that this strain has the ability to reduce eosinophil recruitment and IgE production, possibly associated with Treg cell induction. This is in agreement with numerous studies, where CD4⁺CD25⁺Foxp3⁺ recruitment produced suppression of allergen-induced airway eosinophilia and mucus hypersecretion (Zuany-Amorim *et al.*, 2002; Boudousquie *et al.*, 2009). In an AD mouse model induced by dermal treatment with house-dust-mite extract and dinitrochlorobenzene, administration of the probiotic product IRT5 inhibited clinical symptoms of AD, reducing IgE levels, increasing Treg cell numbers and upregulating the expression of lymphocyte chemokine CCL1 and CCL22 and their receptors (CCR4, CCR8). The authors suggested that during IRT5 administration, DC maturation to rDC occurs, converting lymphocyte CD4⁺Foxp3⁻ to CD4⁺Foxp3⁺ Treg cells capable of migrating to the inflammation site in response to the chemoattractant produced (Ho-Keun *et al.*, 2009). Therefore, enrichment of Treg cells may reduce T effector inflammation and protect against allergy symptoms in this model.

10.3.3 Inflammatory bowel diseases

IBDs are enteropathology that develop due to the loss of intestinal immune tolerance toward the host's own intestinal microbiota, leading to immune activation with subsequent tissue damage and chronic inflammation (Wen *et al.*, 2004). The origin of IBDs remains unknown; however, it is accepted that environmental factors and variations in genes such as MAG12 (associated with mucosal barrier maintenance), NOD2/CARD15, ATG16L1 (involved in bacterial recognition and defence) and IL13R and IL12B (immune regulation) trigger the onset of the

diseases (Wapenaar *et al.*, 2008). IBD can be subdivided in two idiopathic pathologies, Crohn's disease (CD) and ulcerative colitis (UC). The inflammation in CD can develop along the whole intestinal tract, from ileum to colon, and is characterized by a Th1 response with IFN- γ and TNF- α production and tissue-infiltrated Th17 cells (Pene *et al.*, 2008). However, UC is limited to the colon, which becomes inflamed and ulcerated. UC is characterized by an atypical Th2 response, with IL-13 production as well as increased expression of Th17 phenotype (Kobayashi *et al.*, 2008; Shale and Ghosh, 2008).

In the context of these disorders, for example, the intraperitoneal administration of DCs treated with *L. rhamnosus* Lr32 or *L. salivarius* Ls33 has been shown to ameliorate trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice (Foligne *et al.*, 2007b). Intraepithelial $\gamma\delta$ T cells are considered as a T-cell population with regulatory activity against antigens and pathogens, contributing to the maintenance of intestinal homeostasis at epithelial sites; in fact, $\gamma\delta$ T cells have been shown to present regulatory activity in colitis development (Inagaki-Ohara *et al.*, 2004). In a TNBS-induced colitis animal model, a mixture of the strains *L. acidophilus* Bar 1/B. *longum* Bar 33 or *L. plantarum* Bar 10/*St. thermophilus* Bar 20/*B. animalis* subsp. *lactis* was able to prevent colitis. This protective action was associated with an increase in CD4⁺CD25⁺Foxp3⁺ Treg cells and intraepithelial $\gamma\delta$ T cells (Roselli *et al.*, 2009). The regulatory and protection activity was compatible with the cytokine profile produced by probiotic treatment, characterized by a decrease in IFN- γ , TNF- α , MCP1 and IL-12 and by an increase in IL-10. These findings are consistent with the fact that low levels of Treg in peripheral blood have been detected in IBD patients (Maul *et al.*, 2005) and, therefore, the migration of Treg to the inflammation site could be an IBD management strategy.

10.3.4 Autoimmune diseases

Autoimmune diseases are the result of failures in the appropriate function of the immune system, leading to the loss of self-tolerance and abnormal reactivity against the host's own antigens (Schwartz, 2005). The IL-17 pathway is involved in the pathogenesis of many human autoimmune diseases, including rheumatoid arthritis, multiple sclerosis and psoriasis. The IL-17 pathway mediates many of the key tissue-disruptive processes in these diseases, for example bone and cartilage erosion in rheumatoid arthritis and demyelination in multiple sclerosis (Hu *et al.*, 2010).

Although studies in this field are limited, some potentially probiotic strains have proven to exert beneficial effects on prevention or treatment of autoimmune diseases in animal models of multiple sclerosis, which is one of the most important neurological diseases. Multiple sclerosis represents an inflammatory autoimmune disorder against myelin or oligodendrocytes in the central nervous system, probably mediated by T cells. A mixture of the strains *L. paracasei* DSM 13434, *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313 was evaluated in a model of multiple sclerosis and successfully suppressed and reversed chronic encephalomyelitis induced in mice (Lavasani *et al.*, 2010). Suppressor activity was characterized

by an anti-inflammatory cytokine profile, with increased levels of IL-10 and decreased levels of TNF- α , IFN- γ and IL-17 in spleen, MLN and blood. Adoptive transfer assays permitted the association of suppression with IL-10-producing cells CD4⁺CD25⁺Foxp3⁺. Rheumatoid arthritis is characterized by chronic inflammation of the synovial membrane and infiltration of lymphocytes and macrophages, which cause progressive destruction of cartilage and bone (Smolen and Steiner, 2003). In experimental rheumatoid arthritis induced by type-II collagen in animals, administration of a strain of *L. casei* suppressed clinical symptoms, paw swelling, lymphocyte infiltration and destruction of cartilage tissues. These improvements were associated with decreased inflammatory cytokine production, including IL-1, IL-2, IL-6, IL-12, IL-17, IFN- α and TNF- γ .

Diabetes type 1 is a T cell-mediated autoimmune disease characterized by a response in which pancreatic insulin-producing β cells are destroyed. Studies using animal models of non-obese diabetic (NOD) mice have shown that the development of diabetes type 1 is MyD88 dependent and that the interplay between the intestinal microbiota and immune system could influence disease development (We *et al.*, 2008). Studies on NOD mice have shown, for example, that the probiotic mixture VSL#3 prevented diabetes by reducing destruction of insulin-producing β -cells. VSL#3 protection was associated with increased infiltration of IL-10-positive mononuclear cells in the pancreas and upregulation of IL-10 mRNA expression. Mononuclear cells producing IL-10 were also increased in Peyer patches and spleen. Adoptive transference of splenocytes from VSL3-treated animals also provided protection on receiving diabetogenic cells (Calcinaro *et al.*, 2005).

10.4 Evidence of the efficacy of probiotics in humans

10.4.1 Infections

The effectiveness of probiotics in the prevention and treatment of gastrointestinal infections has been evaluated in a considerable number of human clinical trials and particularly in infants and young children. For example, the administration of *L. rhamnosus* LGG during rehydration therapy was shown to reduce diarrhoeal duration associated with rotavirus in children (Guarino *et al.*, 1997; Szajewska *et al.*, 2001). Several studies also suggest that *B. lactis* Bb12 alone or in combination with other strains (e.g. *S. thermophilus*) is associated with a reduction in the number of episodes and duration of diarrhoea or with reduction of diarrhoea risk (Saavedra *et al.*, 1994; Chouraqui *et al.*, 2004; Weizman *et al.*, 2005). Other strains (e.g. *L. reuteri* ATCC55730, etc.) have also been evaluated, but the evidence of their possible efficacy is more limited (Weizman *et al.*, 2005).

Antibiotic-associated diarrhoea (AAD) has also been one of the targets of probiotic studies, being an important cause of diarrhoea due to alterations in the intestinal microbiota that allow over-growth of potentially pathogenic bacteria. So far, original studies and several meta-analyses indicate that some probiotics can decrease the incidence of AAD (D'Souza *et al.*, 2002; Szajewska and Mrukowicz,

2005; McFarland, 2006; Szajewska *et al.*, 2007). The probiotics investigated include *L. rhamnosus* LGG, *E. coli* strain Nissle, 1917 and certain strains of *L. reuteri*, *L. casei*, *L. acidophilus*, *Bifidobacterium* spp., *Enterococcus faecium* SF 68 and *Saccharomyces boulardii*, *L. rhamnosus* LGG and *S. boulardii* being the most promising probiotics in ADD prevention. For example, in a study of 202 children receiving oral antibiotics, 8% of the children who received *L. rhamnosus* LGG concurrently with antibiotics experienced diarrheal symptoms, compared with 26% of the placebo group (Vanderhoof *et al.*, 1999). Similar results were obtained with *S. boulardii* administration to adults, who also showed a decrease in ADD risk (McFarland *et al.*, 1994). In the same study, *S. boulardii* administration was shown to decrease the incidence of antibiotic-associated *Clostridium difficile* infection. In hospitalized elderly patients, a combination of strains of *L. casei*, *L. bulgaricus* and *S. thermophilus* was also shown to reduce AAD risk (Hickson *et al.*, 2007).

Necrotizing enterocolitis has also been a target for probiotic intervention because it is a devastating intestinal disorder affecting premature infants, associated with immature immune system and barrier function or with imbalances in the microbiota causing pathogenic infections (Mackie *et al.*, 1999). In this context, several studies employing probiotic interventions have shown beneficial effects. For example, in a randomized, placebo-controlled study of 40 infants between six and 36 months of age hospitalized with acute diarrhoea (75% associated to rotavirus infection), treatment with high doses of *L. reuteri* SD2222 for up to five days reduced the duration of watery stools compared to the placebo groups (Shornikova *et al.*, 1997). In a six-week multicentre study, low birth weight preterm infants were enrolled and treated with a mixture of two probiotic strains, a *Bifidobacterium bifidum* and a *Lactobacillus acidophilus*. This probiotic treatment reduced the incidence of death or necrotizing enterocolitis (Lin *et al.*, 2008). The most recent meta-analysis, including 11 trials, confirms that some probiotics can reduce death and disease in preterm neonates (Deshpande *et al.*, 2010).

10.4.2 Allergy

Numerous studies have been focused on evaluating the effectiveness of diverse probiotics in prevention and treatment of allergies, since these are very common chronic diseases in infants. A number of randomized controlled trials (RCTs) have been performed to evaluate the role of probiotics in prevention of respiratory allergies, such as rhinitis and asthma, or skin allergies, such as eczema and atopic sensitization. Examples of representative studies are summarized in Table 10.2. Strains of *L. rhamnosus*, *L. acidophilus*, *B. lactis* or *B. breve* have been the most commonly used in preventive studies. Although data are still conflicting, certain probiotic strains seem to contribute to preventing atopic eczema but not sensitization against food allergens. So far, effects of probiotics on prevention of respiratory allergies have not been consistent (Table 10.2).

Fewer trials have been conducted to evaluate probiotic treatment in asthmatic patients, both adults and children. A single probiotic *L. rhamnosus* LGG (Rose *et*

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Table 10.2 Examples of evaluation of probiotic efficacy in the prevention of allergic disease

Reference	Study group	Strain and administration pattern	Positive outcomes	Negative outcomes
Kalliomaki <i>et al.</i> , 2001; Kalliomaki <i>et al.</i> , 2007	159 pregnant Finnish women with family history of allergic disease	<i>L. rhamnosus</i> LGG (2×10^{10} CFU/ml) administered to mothers for 2–4 weeks before delivery and to infants for 6 months after delivery	50% reduction in eczema at 2 years, Ig-E indepen. Subgroup breastfed, protection against eczema at 4 and 7 years	Increase in rhinitis and asthma frequency at 7 years
Niers <i>et al.</i> , 2009	156 women pregnant with children at high risk of developing allergy	<i>B. bifidum</i> W23, <i>B. lactis</i> W52 <i>L. lactis</i> W58, (3×10^9 CFU/dose) administered to mothers for 6 weeks before delivery and to infants for 12 months after delivery	Reduction of eczema proportion at age of 3 months	Probiotics group tended to be more frequently sensitized to food allergens
Kukkonen <i>et al.</i> , 2007	1223 women pregnant with children at high risk of developing allergy	<i>L. rhamnosus</i> LC705 (5×10^9 CFU), <i>B. breve</i> Bb99 (2×10^8 CFU), <i>P. freudenreichii</i> subsp. <i>shermanii</i> JS (2×10^9 CFU) administered to mothers for 2–4 weeks before delivery and to infants for 6 months after delivery	Reduction in eczema frequency	No protection against allergic diseases sensitization at 2 years

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Wickens <i>et al.</i> , 2008	512 pregnant women with a family history of allergic diseases	<i>L. rhamnosus</i> HN001 (6×10^9 CFU/day) or <i>B. animalis</i> subsp. <i>lactis</i> HN019 (9×10^9 CFU/day) administered from 35 weeks of gestation until 6 months before delivery (mother) and to infants for 2 years after delivery	<i>L. rhamnosus</i> group showed reduction in eczema risk	<i>B. animalis</i> had no effect on prevention of eczema or atopy
Huurre <i>et al.</i> , 2008	171 infants and their mothers	<i>L. rhamnosus</i> LGG and <i>B. lactis</i> Bb12 (both 10^{10} CFU/day) to mothers from first term of pregnancy until delivery. Infants only received maternal milk	In a subgroup of infants of allergic mothers, at 1 year old, reduction of risk of atopic sensitization in the probiotic group	No eczema prevention
Kim <i>et al.</i> , 2010	112 pregnant women with a family history of allergic diseases	<i>B. bifidum</i> BGN4, <i>B. lactis</i> AD011 and <i>L. acidophilus</i> AD031 administered at 4–8 weeks before delivery and continuing until 6 months after delivery. Infants were fed breastmilk from 0 to 3 months and then given formula with the probiotic mixture until 4–6 months of age.	Decrease in prevalence and proportion of individuals that develop eczema during 1st year	No difference in total IgE levels or sensitization against food allergens

al., 2010) or mixtures of strains of *S. thermophilus*, *L. bulgaricum* and *L. acidophilus* (Wheeler *et al.*, 1997) or *L. casei*, *L. bulgaricus* and *S. thermophilus* (Giovannini *et al.*, 2007) were evaluated as therapeutic agents in independent studies of asthmatic subjects. However, no treatment was shown to be effective in ameliorating clinical parameters, such as asthma symptoms, quality-of-life or pulmonary function tests. Neither changes in total IgA, IgG, IgM, IgE nor specific IgE, eosinophils or TGF- β , IL-2, IL-4 levels were associated with the probiotic treatment. The administration of milk fermented by *L. acidophilus* strain L-92 to patients with perennial allergic rhinitis, associated with Japanese cedar pollinosis (JCP), resulted in a significant improvement in nasal symptom–medication scores. However, no changes in immune-related parameters (specific IgE levels, T helper Th1/Th2 ratio, eosinophil count, etc.) were detected (Ishida *et al.*, 2005). Administration of milk fermented with *L. casei* strain *Shirota* or a mixture of *L. GG* and *L. gasseri* TMC0356, decreased the mean symptom score for nasal blockage in patients with JCP (Tamura *et al.*, 2007; Kawase *et al.*, 2009), demonstrating that certain probiotic preparations might alleviate some allergy symptoms.

The role of probiotic supplementation has also been evaluated in the treatment of infants with mild-to-moderate atopic eczema, associated with cow's milk allergy. Infants (mean age 4.6 months) were weaned on extensively hydrolysed formula (eHF) with *B. lactis* Bb-12, on this formula with *L. rhamnosus* LGG, or a formula without probiotic strains. In all cases conventional treatment was carried out with emollients and topical corticosteroids. Both probiotics improved the dermatitis score at two months compared to the non-probiotic group, achieving complete resolution of atopic eczema in all groups at six months (Isolauri *et al.*, 2000). In other studies, conventional atopic dermatitis treatment was complemented with administration of *L. rhamnosus*, 19070 and *L. reuteri* DSM 122460 (Rosenfeldt *et al.*, 2003); *L. rhamnosus* and *B. lactis* (Sistek *et al.*, 2006) or *L. fermentum* VRI-003 (Weston *et al.*, 2005). However, probiotics were not effective in resolution of dermatitis in comparison with a placebo. A recent systematic review including 12 trials shows conflicting results of the effects on probiotics on the treatment of atopic eczema, and concluded that their use cannot be generally recommended (Boyle *et al.*, 2009).

10.4.3 Inflammatory bowel diseases

The use of probiotics in IBDs has also been thoroughly investigated in human trials. Of the probiotics tested, *E. coli* strain Nissle and VSL#3 have been shown to be the most effective in preventing recurrence of these disorders. Moreover, VSL#3 prevented recurrence of chronic relapsing pouchitis in two double-blind placebo-controlled trials (Gionchetti *et al.*, 2000; Mimura *et al.*, 2004). VSL#3 also proved effective in prevention of pouchitis in patients after surgery, improving life quality and decreasing pouchitis incidence (Gionchetti *et al.*, 2003). In a RCT, Ishikawa *et al.* (2004) reported that fermented milk containing live bifidobacteria (Yakult strains of the species *B. breve*, *B. bifidum* and *L. acidophilus*) was beneficial in promoting UC remission. In an observational study,

Gosselink *et al.* (2004) reported an association between the administration of *L. rhamnosus* GG and decreased ratio of patients developing pouchitis compared with placebo historical statistics. However, Kuusma *et al.* (2003) did not find differences in mean pouchitis scores between placebo and *L. rhamnosus* LGG-treated groups at the end of a three-month study period. The *E. coli* strain Nissle was also shown to be as effective as mesalazine in UC remission (Rembacken *et al.*, 1999; Kruis *et al.*, 2004). Nevertheless, none of the studies conducted on more severe active forms of IBD provided satisfactory evidence for probiotic effectiveness in the treatment of these disorders (Mallon *et al.*, 2007).

10.5 Conclusion

The intestinal microbiota and some probiotics are known to interact with the host's immune system. These interactions contribute to immune response activation against dangerous stimuli, as well as to the activation of regulatory mechanisms that avoid uncontrolled inflammation and chronic pathologies. Human clinical trials have provided evidence on the efficacy of specific probiotic strains in different conditions, such as prevention of infections and atopic eczema, and the treatment of chronic inflammatory bowel disorders. Nevertheless, evidence on the effectiveness of probiotics in certain conditions cannot be generalized to every strain or strain combination. In many cases, proof of the clinical efficacy of probiotics has been compromised by flaws in the planned trials (e.g. non-controlled, small sample size, etc.) and the lack of comparable studies. In the case of autoimmune disorders, only preclinical trials have been conducted so far. Therefore, well-designed intervention trials are required to consistently support most of the proposed probiotic uses in humans, as well as to make progress in the development of new applications.

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11

Impacts of prebiotics on the immune system and inflammation

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Abstract: The human gut is the natural habitat for a diverse and dynamic microbial ecosystem having an important impact on health and disease. Unabsorbed portions of food are a major nutritional source for these microbial communities. Prebiotics are non-digestible food ingredients that are selectively fermented in the gut resulting in specific changes in the composition or activity of the gastrointestinal microbiota, thus conferring benefits upon host health. Inulin-type fructans and galacto-oligosaccharides are the most extensively studied prebiotics. They are fermented in the colon to short-chain fatty acids, and selectively stimulate the growth of bifidobacteria. These changes have an impact on the mucosal and systemic immune system and are correlated with a series of health benefits reviewed in this chapter.

Key words: galacto-oligosaccharides, immune system, intestinal inflammation, inulin, metabolic syndrome, oligofructose, short-chain fatty acids.

11.1 Introduction

Human beings are associated with a huge population of micro-organisms that live in body surfaces and cavities connected with the external environment. The gut is the natural habitat for the intestinal microbiota, a diverse and dynamic microbial ecosystem including bacteria, archaea and eukarya, which have adapted to live on the mucosal surfaces or within the gut lumen (Guarner and Malagelada, 2003; Ley *et al.*, 2006). The overall interaction between intestinal microbiota and their host is a symbiotic relationship, defined as mutually beneficial for all partners. The host provides a nutrient-rich habitat (Hooper *et al.*, 2002), and the microbial communities confer important benefits on the host's health, including metabolic, protective

and trophic functions (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006).

There is ample evidence indicating that some microbial species living in the gut are potential pathogens and may cause disease when the integrity of the mucosal barrier is functionally breached. However, some other microbial species have never been incriminated in human disease (Borriello *et al.*, 2003). Since the pioneering conception by Nobel Laureate Elie Metchnikoff in the early 20th century (Metchnikoff, 1907), experts have repeatedly suggested that 'a healthy or balanced gut microbiota is one that is predominantly saccharolytic and comprises significant numbers of bifidobacteria and lactobacilli' (Cummings *et al.*, 2004). Taking into account that food ingested by the host is a major nutritional source for the microbial communities living in the gut, it is therefore plausible that unabsorbed portions of food may influence activities and composition of the microbial ecosystem.

Research in the 1980s and 1990s demonstrated that specific non-digestible oligosaccharides were selectively fermented by bifidobacteria and had the capacity of increasing bifidobacteria counts in human faeces (Mitsuoka *et al.*, 1987; Gibson *et al.*, 1995). These observations led to the introduction of the concept of prebiotics by Gibson and Roberfroid (1995). Prebiotics were initially defined as 'non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health'. Complex polysaccharides such as dietary fibre are not hydrolysed by human digestive enzymes but should not necessarily be ascribed to the prebiotic concept as they may not have selective effects on the gut microbiota. For this and other reasons, the authors updated the prebiotic concept some years later, and proposed three criteria for classifying a food ingredient as a prebiotic. Accordingly, (i) a prebiotic should not be hydrolysed by human intestinal enzymes, (ii) it should be selectively fermented by beneficial bacteria, and (iii) this selective fermentation should result in beneficial effects on health or wellbeing of the host (Gibson *et al.*, 2004). The authors refined the definition and proposed that a prebiotic is 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health' (Gibson *et al.*, 2004).

In the years since 2000, the prebiotic concept has attracted the interest of many academic as well as industrial scientists and it has become a popular research topic in nutrition and, more recently, in the biomedical fields. A large part of the research activity has concentrated on the selective stimulation of growth of species from the *Bifidobacterium* and *Lactobacillus* genera. In the future, it is likely this may be expanded towards other genera including *Eubacterium*, *Faecalibacterium* and *Roseburia* (Roberfroid *et al.*, 2010). Research of the Human Microbiome Projects (<http://www.human-microbiome.org>) has contributed towards an improved understanding of the complexity of the gut microbiota (Qin *et al.*, 2010). Recent advances include the discovery of new genera, more accurate information about microbial composition and relative concentration of dominant species in the gut microbiota, their key role on specific health outcomes, and the changes associated with ageing or chronic diseases. As a result of discussions with both academic and

Table 11.1 Description of products with established prebiotic effect**Inulin-type fructans***Linear β (2 $\rightarrow$ 1) fructosyl-fructose polymers**Glucose-fructose_n and/or fructose-fructose_n*

Short to large size polymers (DP 2–60)	Inulin (especially chicory inulin) (DP _{av} 12)
Short oligomers (DP 2–8)	Fructo-oligosaccharides, FOS; scFOS (enzymatic synthesis from sucrose) (DP _{av} 3–6)
	Oligofructose (enzymatic partial hydrolysis of inulin) (DP _{av} 4)
Large size polymers (DP 10–60)	High molecular weight inulin (physical purification) (DP _{av} 25)
	lcFOS
Mixture (DP 2–8) + (DP 10–60)	Mixture of oligomers and large size polymers

Galactans*Mixture of β (1 $\rightarrow$ 6); β (1 $\rightarrow$ 3); β (1 $\rightarrow$ 4) galactosyl-galactose*

Disaccharide	Lactulose
Galactosyl-fructose	
Short oligomers	Galacto-oligosaccharides, GOS
Galactose _n -galactose, and/or galactose _n -gulucose (DP 2–8)	<i>Trans</i> -galacto-oligosaccharides, TOS (enzymatic transgalactosylation of lactose)
Mixture of galactans and inulin-type fructans	Galacto-oligosaccharides and high molecular weight inulin, usually known as GOS-FOS or scGOS-lcFOS
GOS-FOS	

DP, degree of polymerization; DP_{av}, average degree of polymerization; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; lcFOS, long-chain fructo-oligosaccharides; ITF, inulin-type fructans; scFOS, short-chain fructo-oligosaccharides; scGOS, short-chain galacto-oligosaccharides; TOS, *trans*-galacto-oligosaccharides.

Source: adapted from Roberfroid *et al.* (2010).

industry experts appointed by ILSI Europe, a recently published document (Roberfroid *et al.*, 2010) expands the original idea of the prebiotic concept, as: ‘the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host’. Selectivity is the key condition that needs to be demonstrated in studies *in vivo* by applying validated methodologies. It is implicit that the health benefit must be linked or correlated to the selectively stimulated growth of micro-organisms in the gut microbiota.

11.2 Natural sources and prebiotic products

The majority of the scientific data on prebiotic effects have been obtained using food ingredients belonging to two chemical groups, namely inulin-type fructans

and the galacto-oligosaccharides (see Table 11.1). Inulin-type fructans are the most extensively studied prebiotics, and consist of polymers of fructose linked by β 2-1 fructosyl–fructose bonds. A starting glucose moiety can be present but it is not necessary. The galacto-oligosaccharides consist of a lactose core with one or more galactosyl residues linked via β (1–3), β (1–4) and β (1–6) bonds.

These products have repeatedly demonstrated the capacity to selectively stimulate the growth of bifidobacteria and, in some cases, lactobacilli leading to a significant change in gut microbiota composition (Roberfroid *et al.*, 2010). Concurrently, a number of health benefits associated with oral consumption of these substances have been demonstrated. The following list summarizes effects observed in human and animal studies testing inulin-type fructans or galacto-oligosaccharides.

- improvement and/or stabilization of gut microbiota composition;
- improvement of intestinal motor function (stool bulking, stool regularity, stool consistency);
- increase in mineral absorption and improvement of bone health (bone calcium content, bone mineral density);
- modulation of gastrointestinal peptides production, energy metabolism and satiety;
- reduction of blood lipids (triacylglycerols);
- regulation/modulation of immune function;
- improvement of intestinal barrier functions and reduction of metabolic endotoxaemia;
- reduction of risk of intestinal infections;
- reduction of risk of obesity, type II diabetes and metabolic syndrome;
- reduction of risk and/or improvement in the management of intestinal inflammation;
- reduction of risk of colon cancer.

These studies have been extensively reviewed in a handbook (Gibson and Roberfroid, 2008).

Inulin-type fructans are naturally present as an energy reserve in a wide variety of plants (Table 11.2). These are cereals such as wheat and barley, vegetables like onion and garlic, and fruits such as banana and tomato. Such edible plant products represent a natural source of prebiotics in western-style diets (Van Loo *et al.*, 1995). The daily per capita intake is estimated to range from 1–10 g of inulin-type fructans, depending on geographic, demographic and other related parameters (age, sex, season, etc.). Inulin-type fructans content in foods is not measurable by classic methods of dietary fibre analysis and consequently this information may not be mentioned in food tables (Van Loo *et al.*, 1995). Inulin is also present in plants that are not commonly used in human diets (Table 11.2). For instance, high amounts are found in chicory plants, particularly in their roots (16–18% by weight), which makes them a favourite source for commercial exploitation.

Commercially available galacto-oligosaccharides are produced from lactose, but some of them are naturally present in the human milk. The characteristic

Table 11.2 Inulin-type fructans content of miscellaneous plants

Plant	Content (g per 100 g)
Globe artichoke	2–7
Banana	1
Barley	0.5–1
Chicory	15–20
Dandelion	12–15
Garlic	16
Jerusalem artichoke	17–20.5
Leek	3–10
Onion	1–7.5
Wheat	1–4
Salsify	20

Source: adapted from Roberfroid (2005).

composition of human milk is associated with a bifidogenic effect in breastfed neonates (Coppa *et al.*, 2004). The human milk is rich in oligosaccharides that are resistant to digestive processes and reach the colon. Cow's milk and human milk have significant qualitative and quantitative differences regarding these carbohydrates, and the bifidogenic effect of human milk is not observed in infants fed with cow's milk-based formulas. The human milk oligosaccharides comprise a complex mixture of over 130 different types, at a concentration of 10 to 20 g/l (Coppa, 2004; Bode, 2009). The main quantitative component is made up of 25–30 oligosaccharides composed of 3–10 monosaccharide units (glucose, galactose, N-acetylglucosamine, fucose or sialic acid). Characteristically, the monosaccharides are bound by linkages resistant to digestion by human enzymes, including links β (1–3), β (1–4) and β (1–6), etc. Because of their peculiar chemical structure, human milk oligosaccharides have a very significant role in modulating the intestinal microbiota of neonates.

Other oligosaccharides are also suggested as having a prebiotic effect. For example, soybean oligosaccharides, which are composed of galactosyl residues-linked α 1–6 to a sucrose core, are used as prebiotics in Japan. Likewise, isomalto-oligosaccharides are composed of glucosyl residues linked by α (1–6) bonds. These are partially hydrolysed by human enzymes but at a slow rate, and may reach the colon. Xylo-oligosaccharides are also used as functional food in Japan. For all these products, data are promising but further research is required in order to fulfil the criteria for prebiotic classification (Gibson *et al.*, 2004).

11.3 Prebiotic effects in the gut: fermentation and physiology

Certain bacteria of the human gut microbiota are associated with toxin formation and pathogenicity when they become dominant. Knowledge on overt or latent pathogens has advanced markedly, due to their ability to translocate to the bloodstream or other body sites, the infections or clinical complications they can

cause, or the virulence factors they carry. However, there is less consensus regarding what characterizes potentially healthy bacteria. Several beneficial features of gut bacteria are widely recognized, including production of short-chain fatty acids (SCFAs), vitamin synthesis, secretion of defensins or bacteriocins, and inhibition of pathogens through a multiplicity of mechanisms (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006). One subject of particular research interest is the ability of certain species to stimulate immunological defence systems without inducing inflammatory pathways that can be deleterious for the host. The prebiotic concept is based on the selective stimulation of the host's own beneficial microbiota by providing specific substrate for their growth and metabolism.

Colonic micro-organisms are a principal target for prebiotics. Due to the slow transit time of colonic contents, resident micro-organisms have ample opportunity to degrade available substrates (Flint *et al.*, 2008). These may be derived from either the diet or endogenous secretions. Beneficial effects can be obtained from the metabolic activity of gut bacteria on the available substrates (i.e. fermentation profiles and end products), and from the advantage provided by such substrates to genera/species with antagonist potential against harmful micro-organisms and/or mutualistic interactions with eukaryotic epithelial cells (Hooper *et al.*, 2002).

Substrates that reach the colon include non-digestible carbohydrates, dietary fibres, undigested dietary proteins, other proteins of endogenous origin (enzymes, secretions, desquamated epithelial cells) and mucins, the glycoprotein constituents of the mucus which lines the walls of the gastrointestinal tract. The vast majority of the bacteria in the colon are strict anaerobes and thus derive energy from fermentation. Under normal physiological circumstances, the two main fermentative substrates in the colon are non-digestible carbohydrates and proteins. Lipids are only present at influential levels in patients with severe pancreatic insufficiency. The carbohydrate fraction that reaches the colon is variable and depends largely on the composition of the ingested food, whereas cellular desquamation is the principal source of proteins and is less variable (Egert *et al.*, 2006). The overall amount of non-digestible carbohydrate available for colonic fermentation in subjects on a western diet may vary from 20 to 80 g/day. Endogenous carbohydrates, chiefly from mucins and chondroitin sulphate, only contribute about 3–5 g/day of fermentable substrate. On the other hand, up to 50 g of protein are fermented in the human colon daily (Roberfroid, 2005; Egert *et al.*, 2006).

Carbohydrates are fermented in the colon to SCFAs, mainly, acetate, propionate and butyrate (Cummings, 1981), and a number of other metabolites such as lactate, pyruvate, ethanol, succinate as well as the gases H_2 , CO_2 , CH_4 and H_2S (Levitt *et al.*, 1995). SCFAs acidify the luminal pH which suppresses the growth of pathogens. They also influence intestinal motility (Cherbut, 2003) and contribute towards energy requirements of the host (Cummings, 1981). Acetate is metabolized in human muscle, kidney, heart and brain. Butyrate is largely metabolized by the colonic epithelium where it serves as the major energy substrate as well as a regulator of cell growth and differentiation (Cummings, 1981; Williams *et al.*, 2003).

Proteins reaching and/or produced in the colon are fermented to branched-chain fatty acids such as isobutyrate, isovalerate and a range of nitrogenous and sulphur-containing compounds. Unlike carbohydrate fermentation products which are recognized as beneficial to health, some of the end products of amino acid metabolism may be toxic to the host (e.g. ammonia, amines and phenolic compounds) (Macfarlane *et al.*, 1992).

The proximal colon is a saccharolytic environment with the majority of carbohydrate entering the colon being fermented in this region. In the distal colon, carbohydrate availability decreases, and proteins and amino acids become increasingly important energy sources for bacteria (Macfarlane *et al.*, 1992). Consequently, excessive fermentation of proteins in the distal colon has been linked with disease states such as colon cancer and chronic ulcerative colitis, which generally affect the distal region of the large intestine. Thus, it is recognized as favourable to shift gut fermentation towards saccharolytic activity (Roberfroid *et al.*, 2010). The main saccharolytic species in the colonic microbiota belong to the genera *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus* and *Clostridium*. The main proteolytic species belong to the genera *Bacteroides* and *Clostridium* (Roberfroid *et al.*, 2010).

Inulin-type fructans and galacto-oligosaccharides are non-digestible carbohydrates due to their structure based on mono-sugars linked via β bonds. Hydrolytic activity of human digestive enzymes is on α (1–4) and α (1–6) linkages. However, some gut bacteria species and particularly all members of the genus *Bifidobacterium* are rich in β -glycosidases that liberate the constitutive sugar moieties from oligo- and polysaccharides. Hence, both inulin-type fructans and galacto-oligosaccharides are completely fermented in the colon and are converted into bacteria biomass and SCFAs.

Human trials have repeatedly confirmed the prebiotic effect of inulin-type fructans and galacto-oligosaccharides as evidenced by their ability to change the gut microbiota composition after a short feeding period (Table 11.3). At doses starting from around 5 g/day, these compounds increase the number of faecal bifidobacteria by one or two logarithm orders in most individuals (Roberfroid *et al.*, 2010). It has been observed that the daily dose of a prebiotic does not correlate with the absolute numbers of 'new' bacterial cells that appear as a consequence of the prebiotic treatment. The initial numbers of bifidobacteria seem to influence the prebiotic effect, and those subjects with initial low counts are the best responders after prebiotic feeding (De Preter *et al.*, 2008). Interestingly, changes have also been shown in the mucosa-associated microbiota. In an *ex vivo* protocol in which 15 healthy volunteers selected from a colonoscopy waiting list were asked to supplement their usual diet with inulin (15 g/day) for two weeks, increases in both bifidobacteria and lactobacilli counts in the colonic mucosa were reported (Langlans *et al.*, 2004).

Oligofructose-enriched inulin and lactulose have been shown to reduce microbial fermentation of proteins in the human colon (De Preter *et al.*, 2007, 2008), as a consequence of enhanced saccharolytic activity. Inulin-type fructans have positive effects on basic physiological functions of the colon, i.e., stool production and faecal excretion (Roberfroid, 2005), and are being used for bowel regularity.

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Table 11.3 Prebiotic effect of inulin-type fructans and galacto-oligosaccharides in human studies

Prebiotic	Subjects	Dose	Duration	Effect
Inulin	8 healthy adults, placebo-controlled	34 g/d	64 days	Significant increase in bifidobacteria established by FISH
scFOS	40 healthy adults	2.5–20 g/d	14 days	Significant increase in bifidobacteria without excessive gas production
Inulin and FOS	4 or 8 healthy adults	15 g/d	45 days	Bifidobacteria becoming predominant in faeces with both inulin and oligofructose
Inulin	35 constipated elderly	20 and 40 g/d	19 days	Significant increase in bifidobacteria, decreases in enterococci and fusobacteria
FOS in biscuits	31 healthy adults, double-blind placebo-controlled	7 g/d	42 days	Significant increase in bifidobacteria established by FISH, no change in total bacteria levels
FOS	12 healthy adults	4 g/d	42 days	Significant increase in bifidobacteria, no change in total bacteria levels
FOS	8 healthy adults, placebo-controlled	8 g/d	5 weeks	Significant increase in bifidobacteria and decrease in faecal pH
GOS	12 healthy adults	15 g/d	7 days	Significant increase in faecal lactic acid bacteria (culture)
GOS plus FOS	90 term infants, placebo-controlled	0.4 and 0.8 g/d	28 days	Dose-dependent increase in bifido bacteria and lactobacilli, and softer stools
scFOS or GOS	40 healthy adults, double-blind, parallel group	10 g/d	6 weeks	Significant increase in bifidobacteria
scFOS	12 healthy elderly	8 g/d	4 weeks	Well tolerated and significant increase in bifidobacteria
Inulin	14 healthy adults	9 g/d	2 weeks	FISH probes show increased bifidobacteria
Inulin	45 healthy adults	7.7 then 15.4 g/d	3 weeks	Increased bifidobacteria and decreased bacteroides
Inulin	40 healthy adults	8 g/d	2 weeks	Significant increase in bifidobacteria established by FISH
Inulin/FOS	19 healthy adults	10 g/d	4 weeks	Significant increase in bifidobacteria
scFOS	19 healthy elderly	8 g/d	3weeks	Significant increase in bifidobacteria
scFOS	10 healthy adults	4 g/d	2 weeks	Significant increase in bifidobacteria and lactobacilli
Inulin	30 healthy adults	5 or 8 g/d	2 weeks	Both doses increased bifidobacteria, a higher percent of volunteers responded to 8 g/d
GOS	30 healthy adults	3.6 or 7g/d	7 days	Selective bifidogenic effect

FISH, fast *in situ* hybridization.

Source: adapted from Roberfroid *et al.* (2010).

11.4 Prebiotic effects and the immune system

Studies in germ-free and gnotobiotic animals have clearly documented the critical role of the microbiota for an optimal structural and functional development of the immune system (Yamanaka *et al.*, 2003; Gaboriau-Routhiau *et al.*, 2009; Round and Mazmanian, 2009). Animals bred in a germ-free environment show low densities of lymphoid cells in the gut mucosa and low levels of serum immunoglobulins. Exposure to commensal microbes rapidly expands the number of mucosal lymphocytes and increases the size of germinal centres in lymphoid follicles (Yamanaka *et al.*, 2003). Subsequently, immunoglobulin-producing cells appear in the lamina propria and there is a significant increase in serum immunoglobulin levels. Most interestingly, recent findings suggest that commensals play a major role in the induction of regulatory T cells in gut lymphoid follicles (Round and Mazmanian, 2010). These notions are very relevant for a proper understanding of the interactive coexistence of the immune system and the gut microbiota. The gut-associated lymphoid tissue has evolved to provide both optimal defence against intestinal pathogens and tolerance towards dietary antigens and commensal non-pathogenic microbes (Fig. 11.1).

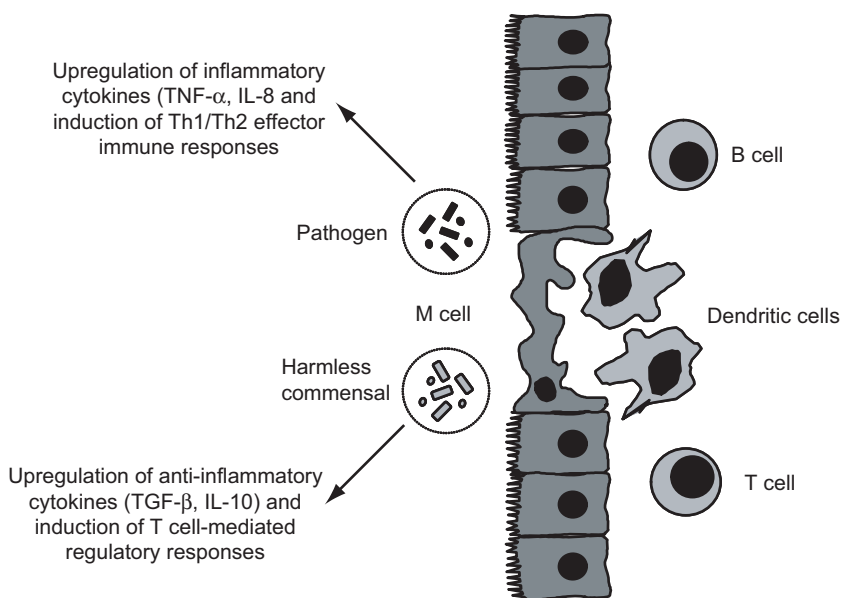


Fig. 11.1 The specialized lymphoid follicles of the gut mucosa are the major sites for induction and regulation of immune responses. The mechanisms which determine the phenotype differentiation of T helper cells are not fully understood, but certainly depend on co-stimulatory signals from the innate immune system. Cytokine release by epithelial cells and/or antigen presenting cells may play a critical role in the induction of effector or regulatory pathways.

Prebiotics may influence the immune system directly or indirectly either via specific products derived from carbohydrate fermentation or by altering microbial composition of the gut microbiota. Increased numbers of a particular microbial genus or species, or relative decrease of other microbial species, may change the collective interaction of the microbiota on immune sensors and shift the balance between effector and regulatory pathways (Guarner *et al.*, 2006). On the other hand, microbial products such as SCFAs, defensins or other bioactive peptides may interact with immunocompetent cells, including enterocytes, and modify their status of activation and/or their activity. For instance, some G protein-coupled receptors for SCFA are expressed on leukocytes, especially in polymorphonuclear cells (Le Poul *et al.*, 2003), as well as on epithelial cells of the human colon (Karaki *et al.*, 2008). SCFAs modulate chemokine expression in intestinal epithelial cells (Sanderson, 2007), and are critical for controlling inflammatory responses and maintaining intestinal homeostasis (Maslowski *et al.*, 2009). Finally, some oligosaccharides including oligofructose can bind to cell receptors on pathogenic bacteria preventing them from attaching to epithelial cells and so avoiding invasion.

Recent comprehensive articles have reviewed in depth the current knowledge on the immunomodulatory potential of prebiotics (Seifert and Watzl, 2007; Lomax and Calder, 2009). As shown in these reviews, studies indicate that feeding inulin-type fructans to mice increases the concentration of total secretory IgA in the intestinal lumen. The number of B lymphocytes in Peyer's patches was shown to increase in parallel to IgA changes. Those studies also showed enhanced interleukin (IL)-10 cytokine secretion by intestinal tissue, and decreased transcription and concentration of pro-inflammatory cytokines (Roller *et al.*, 2004). There may be a greater effect in younger animals as their gut immune system is still developing and may therefore be more susceptible to modulation (Lomax and Calder, 2009).

Prebiotics have been tested in humans for their ability to prevent infectious diseases, as this outcome would be a relevant consequence of improving immune function. In infants aged 6–12 months (87% breast-fed) the intake of oligofructose as part of an infant cereal had no effect on diarrhoea prevalence and on vaccination-induced antibody titres to *Haemophilus influenza* when compared to the infant cereal alone (Duggan *et al.*, 2003). Besides the fact that a rather low dose of oligofructose was supplemented, breastfeeding may already have provided adequate amounts of human milk oligosaccharides in this study. In another study, episodes of common childhood diarrhoea were reported to be reduced in healthy infants supplemented with oligofructose (Waligora-Dupriet *et al.*, 2007). Incidence of acute diarrhoea was also reduced in infants who received an infant formula containing GOS/ FOS (Bruzese *et al.*, 2009). In adults, oligo-fructose supplements were reported useful for decreasing relapse rate of *Clostridium difficile*-associated diarrhoea (Lewis *et al.*, 2005).

A number of human studies tested the effect of prebiotics on various immunological parameters. A study in eight-month-old infants supplemented with inulin-type fructans reported an increase in post-vaccination measles IgG levels in the blood (Firmansyah *et al.*, 2001). Interestingly, early life exposure of non-breast-fed infants to oligosaccharides had an effect on natural immunoglobulin

production (Van Hoffen *et al.*, 2009), as mixtures of GOS/FOS were shown to result in significantly higher faecal secretory IgA concentrations as a consequence of the prebiotic effect (Bakker-Zierikzee *et al.*, 2006; Scholtens *et al.*, 2008). Overall, data on faecal secretory IgA in infants show increments associated with prebiotic consumption, and these findings are consistent with the observations in animal models.

In addition to effects on integrated immune responses, the prebiotic effect on specific immune markers has also been tested (see Lomax and Calder, 2009; Roberfroid *et al.*, 2010). A well-designed and controlled human intervention study investigated the effect of a mixture of galactans on the immune system of healthy elderly volunteers. This study reported that intake of galactans (5.5 g/day) for 10 weeks significantly increased phagocytosis, natural killer (NK) cell activity and the production of the anti-inflammatory cytokine IL-10, while the production of pro-inflammatory cytokines IL-1 β , IL-6, and tumour necrosis factor (TNF)- α was reduced (Vulevic *et al.*, 2008). A clear positive correlation between numbers of bifidobacteria in faecal samples and both NK cell activity and phagocytosis was observed. Again, these data are consistent with the ability of prebiotics to favour regulatory pathways, as also suggested by similar observations in animal experiments. These findings provide some light about plausible mechanisms of action for the beneficial effects of GOS/FOS in infants at risk of allergies, as one study reported a reduction in the incidence of atopic dermatitis (Moro *et al.*, 2006).

In summary, human and animal data suggest that inulin-type fructans and galactans may potentially affect the immune system as a direct or indirect result of the change in the composition and/or fermentation profile of the gut microbiota. Several studies have shown increased secretory IgA levels as a result of prebiotic consumption. The immunomodulatory effects may result in clinically relevant outcomes such as improved resistance against enteric infections or prevention of allergies.

11.5 Intestinal inflammation and the metabolic syndrome

11.5.1 Intestinal inflammation

There is convincing evidence that intestinal inflammation results from the interaction of the gut microbiota with the mucosal immune compartments. The gastrointestinal tract is adapted to the analytical recognition of the external environment. The large mucosal surface is a sensitive interface that includes tools and structures allowing a detailed scrutiny of foreign bodies transiting along the tract. From a functional point of view, gut-associated lymphoid tissues generate either immuno-inflammatory responses for rejection of potential pathogens, or non-inflammatory responses for tolerance towards dietary antigens and commensal non-pathogenic microbes (Fig. 11.1). In pathological conditions such as the inflammatory bowel diseases, abnormal communication between gut microbial communities and the mucosal immune system is being incriminated as the core defect leading to mucosal lesions (Strober *et al.*, 2007). Either primary dysregulation of the mucosal immune system leads to excessive immunologic responses to

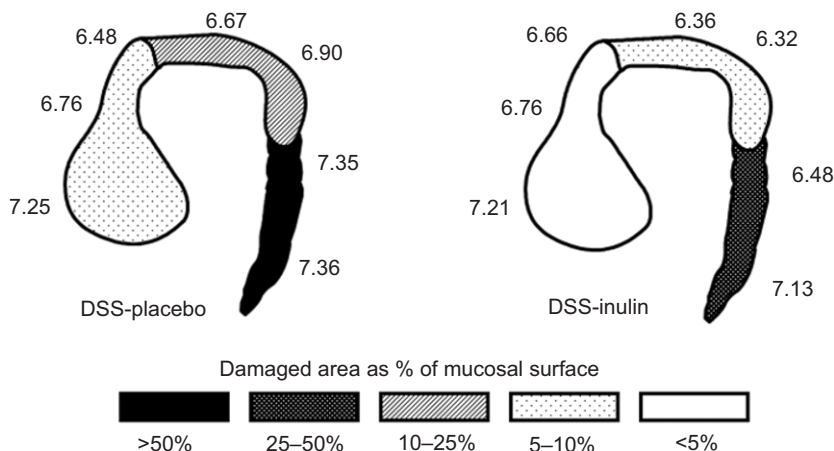


Fig. 11.2 Comparative layout of the colonic mucosal surface (caecum to rectum) of rats with DSS-induced colitis that were treated with either oral inulin or placebo for five days. Mean intraluminal pH recordings are shown by the adjacent figures and mucosal lesion scores are represented by patterns. Areas of highly damaged mucosa were found at distal segments of the colon (rectum). Microbial fermentation generates short chain fatty acids, inducing an acidic environment within the colonic lumen. Inulin feeding extended the area of acidic environment from right to distal colon, and acidic pH was associated with attenuated scores of mucosal damage. Data from Videla *et al.* (2001).

normal microbiota, or changes in the composition of gut microbiota elicit pathologic responses from a normal mucosal immune system. In either case, intestinal inflammation is driven by the gut microbiota (Sartor, 2008).

Despite the evidence that the microbiota is necessary to drive the inflammation, certain microbial species may indeed protect the mucosa from inappropriate host-damaging inflammatory responses. Studies in both animal models and with inflamed mucosal samples from patients with inflammatory bowel disease (IBD) have shown that some specific bacteria can downregulate the expression of a number of key pro-inflammatory cytokines (TNF- α , interferon (IFN)- γ , IL-6, IL-23p19, IL-12p35, IL-17F), and chemokines (IL-8, CXCL1, CXCL2) (Borruel *et al.*, 2002; Carol *et al.*, 2006; Foligne *et al.*, 2007; Llopis *et al.*, 2009), thereby reducing the mucosal lesion scores in the animal models of intestinal inflammation (Llopis *et al.*, 2005; Foligne *et al.*, 2007). Moreover, some probiotic species including bifidobacteria are able to stimulate immunoregulatory IL-10 production (Borruel *et al.*, 2003; Hart *et al.*, 2004). Thus, some components of the gut microbiota may induce immuno-regulatory pathways that mitigate inflammatory reactions.

Prebiotic stimulation of microbial species with anti-inflammatory properties would help in controlling inflammatory processes of the intestinal mucosa. Numerous experimental studies have been conducted to investigate the impact of prebiotics on chronic intestinal inflammation in animal models of IBD. In the rat

model of colitis induced by the chemical dextran sodium sulphate (DSS), oral administration of inulin reduced significantly tissue myeloperoxidase activity, an index of neutrophil infiltration, and mucosal release of inflammatory mediators (Videla *et al.*, 2001). Furthermore, inulin-fed rats showed a reduced area of mucosal damage and decreased severity of crypt destruction (Fig. 11.2).

The effect of oligofructose has also been tested in rats with experimental colitis induced by the hapten trinitrobenzene sulphonic acid (TNBS) (Cherbut *et al.*, 2003). Oral administration of oligofructose significantly reduced intracolonic pH, macroscopic lesion scores and tissue myeloperoxidase activity in rats with TNBS colitis. In addition, oligofructose increased the concentration of lactate and butyrate as well as counts of lactic acid bacteria in caecal contents.

Further experimental work evaluated the anti-inflammatory effects of inulin and oligofructose in the transgenic HLA-B27 rat model of spontaneous colitis (Hoentjen *et al.*, 2005). Rats transgenic for the human HLA-B27- β 2-microglobulin gene spontaneously develop immune-mediated colitis of variable severity at two to four months of age. The disease is characterized by non-bloody diarrhoea and marked inflammatory infiltration of the caecal and colonic mucosa. Daily treatment with inulin-type fructans reduced gross morphological scores and histological grading of the lesions. Treatment with lactulose was also shown to be effective in IL-10 gene knockout mice which spontaneously develop colitis (Madsen *et al.*, 1999). Taken together, all these experimental data give a strong indication of the anti-inflammatory effects of prebiotics in a wide range of animal models of intestinal inflammation. However, there are only a few trials that have tested prebiotics in the clinical setting.

Pouchitis is a condition characterized by chronic mucosal inflammation of the ileal pouch in patients that had a total colectomy. The ileal pouch is surgically constructed in order to function as a faecal reservoir. A controlled study included 20 patients with active pouchitis that were randomized to consume 24 g/day inulin or placebo for three weeks in a cross-over design (Welters *et al.*, 2002). There was a significant reduction in pouchitis disease activity index during the intervention with inulin as compared with placebo.

Two trials have used ingredients showing a prebiotic effect to investigate their efficacy in the management of ulcerative colitis. The first was a pilot study of 18 patients with mild active disease, who were randomized to receive either a synbiotic (6g/day of oligofructose and *B. longum*) or a placebo. Only 14 patients completed the study and there was no difference in clinical scores between the intervention and control group, but there was a lower degree of inflammation in the intervention group (Furrie *et al.*, 2005). In another pilot study in active ulcerative colitis 19 patients were randomized to receive either a prebiotic (12 g/day of oligofructose and inulin) or placebo, in conjunction with 3 g/day mesalazine for two weeks (Casellas *et al.*, 2007). Only 15 patients completed the study (seven intervention, eight control) and, although there was a reduction in disease activity, this occurred in both groups, potentially due to them both starting concomitant drug therapy. However, compared with placebo, the intervention group had significantly lower concentrations of the inflammatory marker faecal calprotectin.

In Crohn's disease, a large double-blind, randomized, placebo-controlled trial of inulin-type fructans (15 g/day) in 103 patients with active disease found no significant differences in clinical activity scores or the number of patients entering disease remission between groups. However, as the trial has only been presented as a conference abstract there is currently limited information and no microbiological and immunological data published (Benjamin *et al.*, 2010).

Irritable bowel syndrome (IBS) is a functional bowel disorder manifested by chronic, recurring abdominal pain or discomfort associated with disturbed bowel habit, in the absence of structural abnormalities. The symptomatic array may include abdominal pain, discomfort, distension, cramping, distress, bloating, excess flatulence, and variable changes in frequency and form of stools. A precise aetiology for IBS is not recognized. However, epidemiological studies have identified a series of pathogenetic factors including altered response to stress and inflammatory post-infectious processes of the gut mucosa (Spiller *et al.*, 2007).

Two published studies of adequate study design have reported on the effect of prebiotics in IBS. The first study screened 2235 subjects and recruited and randomized 105 patients (50 analysed) with IBS fulfilling Rome II criteria with minor intensity of symptoms as assessed by an initial questionnaire. Treatment with inulin-type fructans at 5 g/day for six weeks reduced incidence and intensity of symptoms as compared to the placebo product. Prebiotic treatment also improved functional digestive disorders related quality of life (Paineau *et al.*, 2008). The second study randomized 60 subjects (44 analysed) according to Rome II criteria into three groups either receiving 7 g/day placebo, 3.5 g/day of GOS and 3.5 g/day placebo, or 7 g/day of GOS, for six weeks. The 3.5 g/day prebiotic treatment significantly improved flatulence, bloating and composite score of symptoms as well as subjective global assessment. It also increased the proportion of bifidobacteria in faecal samples (Silk *et al.*, 2009).

In summary, small pilot studies indicate potential benefit in treating active inflammation in patients with pouchitis or mild ulcerative colitis. One large trial has demonstrated no clinical benefit of treating active Crohn's disease with a prebiotic. Two studies in IBS provided positive outcomes for the prebiotics tested. None of the trials conducted thus far have reported concerns regarding the safety of inulin-type fructans in IBD or IBS, or galactans in IBS.

11.5.2 Metabolic syndrome

Obesity and insulin resistance are associated with low grade systemic inflammation (Cani and Delzenne, 2009). Gut microbial communities seem to play a role in the origin of the inflammatory disturbance associated with the metabolic syndrome. In fact, endotoxin lipopolysaccharides (LPS) of Gram-negative bacteria have been considered to be the triggering factor for the early development of low-grade inflammation leading to insulin resistance (Cani *et al.*, 2007a). Excessive intake of dietary fat facilitates the absorption of pro-inflammatory bacterial LPS from the gut, thereby increasing plasma LPS level leading to subclinical metabolic endotoxaemia. Interestingly, when metabolic endotoxaemia was reproduced in

mice through continuous subcutaneous infusion of LPS for four weeks, fasted glycaemia and insulinaemia, as well as adipose tissue and whole body weight, were increased to a similar extent as in high-fat-fed mice (Cani *et al.*, 2007a). In addition, parallel experiments showed that obesity induced by diet (high-fat feeding) (Cani *et al.*, 2007a) or by genetic deletion (leptin-deficient models) (Waldram *et al.*, 2009) is associated with changes in gut microbiota characterized by a decreased number of bifidobacteria.

Subsequent experiments tested the effect of feeding mice with inulin-type fructans. The prebiotic restored the number of intestinal bifidobacteria and reduced the impact of high-fat diet on metabolic endotoxaemia and low-grade inflammatory disturbance (Cani *et al.*, 2007b, 2009). Prebiotic feeding also improved glucose tolerance and glucose-induced insulin secretion in high-fat diet mice. Data obtained in obese ob/ob mice suggested that treatment with the prebiotic increased production of a gut peptide secreted by endocrine cells of the colon, namely glucagon-like peptide-2 (GLP-2), which plays a role on intestinal permeability, by restoring tight junction protein expression. In this model, inulin-type fructans increase the numbers of bifidobacteria in the gut, stimulate secretion of GLP-2, prevent changes in gut permeability, reduce endotoxaemia and mitigate the associated inflammatory disorders as well as the metabolic syndrome (Cani *et al.*, 2009).

The relevance of endotoxaemia in metabolic disorders due to fat excess and diabetes in humans is supported by some recent studies. Indeed, high-fat meals also lead to increased LPS plasma levels in humans (Erridge *et al.*, 2007). Differences in gut microbiota composition may precede the development of becoming overweight (Kalliomaki *et al.*, 2008). This study found that numbers of faecal bifidobacteria during the first year of life were higher in children who exhibited a normal weight at seven years than in children becoming overweight. However, the impact of the prebiotic approach on endotoxaemia and inflammation in obese and diabetic patients has not yet been demonstrated. Intervention studies with prebiotics are necessary to prove the role of gut microbial composition in the onset of insulin resistance and metabolic syndrome in humans.

11.6 The role of prebiotics in human health and clinical nutrition

There is growing interest in understanding the beneficial role of the microbial communities that inhabit the human gut. The intestinal microbiota has a collective metabolic activity equal to a virtual organ within an organ (O'Hara and Shanahan, 2006). The microbiota is perceived as a positive health asset for normal body growth and development, as well as for defence against infection. Gut bacteria crucially influence the normal structural and functional development of the mucosal immune system (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006). The mechanisms underlying the conditioning influence of the bacteria on mucosal homeostasis and immune responses are beginning to be unravelled.

However, most of our current knowledge derives from experiments performed in germ-free animals, and actual impact of particular microbial communities on human health outcomes is still a matter of research. The availability of high-throughput sequencing technologies as well as the development of new bio-informatic tools is providing new insights on the microbiota, not only in terms of diversity in composition but also in terms of the functions provided by the large set of microbial genes harboured within the human gut (Qin *et al.*, 2010). Nonetheless, on the basis of our current knowledge, experts have suggested that a healthy or balanced gut microbiota is one that is predominantly saccharolytic and comprises significant numbers of bifidobacteria and lactobacilli (Cummings *et al.*, 2004).

A large number of human intervention studies have demonstrated that dietary consumption of inulin-type fructans or galactans results in statistically significant changes in the composition of the gut microbiota. Most of the available data concern the selective stimulation of bifidobacteria and, in some studies, also lactobacilli. In addition, investigators have also reported a concomitant reduction in the concentration of pathogenic bacteria such as clostridia and salmonella. Fermentation of prebiotics within the colon is associated with generation of SCFAs and reduction of proteolytic/putrefactive microbial activities. The more data are accumulated, the more it will be recognized that such changes in the composition and activity of the intestinal microbiota can be regarded as markers of intestinal health (Roberfroid *et al.*, 2010).

Specific indications of prebiotics for human health include their use in infant formulas. The effect of breastfeeding on infant gut microbiota composition is well established and mother's milk is known to contain complex mixtures of oligosaccharides with prebiotic bifidogenic effects. Therefore, infant formulas and foods have been supplemented with prebiotics, and such supplementation increases the faecal concentration of bifidobacteria.

Results from experimental models, but also observed in a few human studies, clearly suggest that the prebiotic effect is associated with changes in certain immunological biomarkers and affects the activity of the immune system. Several studies have shown increased secretory IgA levels as a result of prebiotic consumption. Prebiotics may reduce the incidence and duration of certain infections in infants and children. Data published so far suggest that prebiotics may be most beneficial in those who are particularly susceptible to modifications of their immune system (Lomax and Calder, 2009). One study has shown efficacy for the prevention of atopic dermatitis.

Changes in the gut microbiota composition are classically considered as one of the many factors involved in the pathogenesis of either IBD or IBS. Promising beneficial effects have been demonstrated in animal models. Still, data from clinical studies do not show relevant therapeutic value for prebiotics in IBD. However, they may have a role in the nutritional support of IBD patients, and may alleviate symptoms in some subsets of individuals with IBS complaints.

Dietary intake of inulin-type fructans or lactulose has been shown to increase calcium absorption as well as bone calcium accretion improving bone mineral

density, especially in adolescents. This topic was not addressed in this chapter but has been recently reviewed in depth (Roberfroid *et al.*, 2010).

Finally, interesting data, mainly from experimental models, support the beneficial effects of inulin-type fructans on energy homeostasis, satiety regulation and body weight gain. These studies suggest that the gut microbiota composition (especially the number of bifidobacteria) may contribute to modulating metabolic processes associated with the metabolic syndrome. A leading role of altered gut permeability and metabolic endotoxaemia in the pathogenesis of insulin resistance and diabetes has been reported. Prebiotic food ingredients have been shown to reverse these derangements in the animal model.

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12

Food bioactive proteins and peptides: antimicrobial, immunomodulatory and anti-inflammatory effects

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Abstract: In addition to supplying essential nutrients, it is now well accepted that some food proteins can confer additional health benefits beyond nutrition. Some food proteins act directly in their intact form to elicit physiological effects, but generally peptides resulting from digestion, hydrolysis or fermentation of the protein are considered of the most interest, and these frequently display greater bioactivity than the parent protein. In this chapter we focus on the potential for generation and absorption of food bioactive peptides in the gut and their site of action. The range of reported physiological effects of bioactive proteins and peptides is diverse and we review bioactive proteins and peptides with antimicrobial, immunomodulatory and anti-inflammatory effects, their potential mechanisms of action and applications to human health.

Key words: proteins, peptides, bioactivity, antimicrobial, immunomodulatory, anti-inflammatory.

12.1 Introduction

From a nutritional perspective, food proteins were once considered solely as a supply of amino acids to the body. However, it is now well established that, in addition to their nutrient-supply role, some food proteins in their intact form possess bioactive properties and, upon digestion, proteins may give rise to a myriad of bioactive peptides. This is a young field of research, but it is becoming increasingly clear that bioactive peptides, latently encrypted in the primary sequences of proteins, may play an important modulatory role in human body systems.

Bioactive substances have been defined as: ‘food components that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health’ (Schrezenmeir *et al.*, 2000). Moller *et al.* (2008) have refined the latter definition by proposing two caveats:

- (a) ‘To be considered “bioactive”, a dietary component should impart a measurable biological effect at a physiologically realistic level’, and
- (b) ‘The “bioactivity” measured has to have the potential (at least) to affect health in a beneficial way, thus excluding from this definition potentially damaging effects (such as toxicity, allergenicity and mutogenicity, which are undoubtedly a reflection of “bioactivity” in its broadest sense).’

According to the latter definition, both food proteins and peptides may be considered as bioactives. Some food proteins (e.g. lactoferrin, wheat albumin, immunoglobulins) act directly in the intestine in their intact form, but seemingly of even greater importance are the numerous peptides released from proteins by enzymatic activity during digestion, or by hydrolysis or fermentative processes occurring during food processing or ripening.

The aim of this chapter is to provide a brief overview of food bioactive proteins and peptides in general, and to focus on their roles in modulating immunity and inflammation.

12.2 Sources of food-derived bioactive peptides and proteins

With respect to the discovery of bioactive proteins and peptides, milk has been by far the most studied food, and has been found to be a rich source of bioactives. Milk proteins, themselves, may have potent bioactivity, ranging from antimicrobial effects to the facilitation of nutrient absorption, and may act specifically as growth factors, hormones, enzymes, antibodies and immune modulators. Common examples of milk proteins possessing bioactivity as intact proteins are lactoperoxidase, lactoferrin, lysozyme, immunoglobulins and glycomacropeptide. Bioactive properties have also been attributed to some of the major milk proteins such as an antioxidant activity of β -lactoglobulin and immunomodulatory activity of α -lactalbumin. Bovine α -lactalbumin has been shown to stimulate mucus metabolism in rat gastric mucosa (Ushida *et al.*, 2007). Some of the milk proteins (e.g. secretory immunoglobulin A, lactoferrin, 1-antitrypsin, lactalbumin) may be relatively resistant to digestive enzymes, thus allowing the protein to exert its effect in the gut. Lactoferrin and immunoglobulins have been shown to partly escape degradation in the upper digestive tract (Roos *et al.*, 1995; Drescher *et al.*, 1999; Korhonen and Marnila, 2006). It has also been shown experimentally that the milk protein, casein, may confer a protective effect on the digestion of certain milk constituents such as epidermal growth factor, transforming growth factor (TGF)- α , somatostatin and insulin-like growth factor (Rao *et al.*, 1990; Playford *et al.*, 1993; Xian *et al.*, 1995).

Although the bioactivities associated with intact proteins are an important

component of the food modulatory cascade, there is evidence in some cases that partial digestion of the protein leads to an enhanced bioactivity. Native lactoferrin, for example, is bactericidal, but digestion by pepsin of both human and bovine lactoferrin releases antimicrobial peptides (lactoferricin) which have greater potency than the parent protein (Bellamy *et al.*, 1992). The relatively large Fab fragment of immunoglobulin molecules has also been shown to have potent antimicrobial activity (Gorevic *et al.*, 1985). Moreover, the milk proteins act as 'bioactive precursors', giving rise during digestion in the gut to variously sized peptides exhibiting a range of bioactivities (Aimutis, 2004; FitzGerald *et al.*, 2004; Meisel, 2005; Silva and Malcata, 2005).

Peptides from milk with opioid agonist activity (e.g. casomorphins from α - and β -casein; α -lactorphin from α -lactalbumin; β -lactorphin from β -lactoglobulin) have been identified, as have peptides with opioid antagonistic properties (e.g. lactoferroxins from lactoferrin; casoxins from κ -casein). The actions of small angiotensin-converting enzyme (ACE)-inhibitory peptides involved in the control of blood pressure (e.g. casokinins from α - and β -casein; lactokinins from α -lactalbumin and β -lactoglobulin) are well documented. Casein (both α - and β -casein) gives rise to immunomodulatory peptides; lactoferrin to the antimicrobial lactoferricin; κ -casein and transferrin to the antithrombotic casoplatelins and α - and β -casein to mineral-binding and anticariogenic phosphopeptides. A generalised effect of peptides from casein on gut protein secretory and absorptive capacity has been the subject of review (Rutherford-Markwick and Moughan, 2005; Moughan *et al.*, 2007).

When considering the biological functions of milk constituents, it is important to reflect on both their functions in the mammary gland itself and nutritional/developmental functions in the neonate, with some constituents possibly being important in both respects. For example, α -lactalbumin, a calcium-binding protein having the ability to modulate the substrate specificity of lactose-synthase in the lactating mammary gland, also gives rise to several bioactive peptides in the gut lumen. Molecules such as α -lactalbumin can be considered as multifunctional. Lactalbumin has functions in milk synthesis and secretion; it functions as a food for the neonate and further gives rise to peptides upon digestion having antimicrobial and other biological functions.

There is a wealth of evidence that various constituents of colostrum and milk can be absorbed from the gut of the neonate, and thus milk and colostrum constituents undoubtedly play a role in the maturation of the gut and have effects luminally in the gut, locally at the mucosal surface and systemically following absorption (Kelly and Coutts, 1997; Meisel, 2005).

It is not really surprising that milk should be a rich supply of bioactive proteins and peptides. Milk is a 'biological fluid' tailor-made to meet not only the nutritional but also the developmental needs of the new-born infant. What is perhaps more surprising is that diverse physiological effects of milk proteins and milk-derived peptides have been shown not only in infants but also in adults, and what is more remarkable still is that the source of food bioactive proteins and peptides is by no means confined to milk.

Proteins isolated from wheat germ, wheat flour and soya cotyledons have been shown *in vitro* to exert pancreatic lipase inhibitory action (Satouchi *et al.*, 1974; Borel *et al.*, 1989; Tani *et al.*, 1994) and a water extract from defatted rice bran reduced plasma triglyceride levels in rats (Tsutsumi *et al.*, 2000). Proteins such as phaseolamin from kidney beans and wheat albumin have α -amylase inhibitory activity (Shimizu, 2004). Moreover, bioactive peptides have been reported to be generated, at least under *in vitro* conditions, from food proteins as diverse as blood, gelatin, meat, eggs, fish, wheat, maize, soyabean, rice, mushrooms, pumpkin and sorghum (Moller *et al.*, 2008). Indeed at first sight it would appear that the production of bioactive peptides from food proteins is ubiquitous, but not all proteins have been shown to give rise to bioactive peptides and certainly not all proteins generate bioactive peptides in the same amounts.

Have mammals evolved over time to adapt to their food source, such that exogenous food-derived bioactive peptides are an essential supplement/complement to endogenous physiological regulation? The widespread nature of the effects of bioactive peptides from food has led Pellegrini (2003) to conclude that antimicrobial peptides derived from food can be considered as a component of 'nutritional immunity'. Likewise, Teschemacher (2003) has referred to food-derived opioid peptides (opioid receptor ligands) as an 'exogenous supplement to the endogenous opioidergic system of humans' and that 'at least the milk protein-derived opioid peptides are (more than likely) essential supplements of the opioidergic system'. Whereas it is clear that bioactive peptides derived from food do have modulatory effects in the human adult, whether they can be regarded as essential for well-being is questionable. The types and amounts of protein consumed by humans are not consistent. They vary from day to day and season to season, and individuals may go for long periods of time without consuming certain proteins or mixtures of proteins. Having said this, bioactive peptides derived from food are numerous, often having over-lapping functional activities and targets, and with some individual peptides being multifunctional. These features of multiplicity of action do imply some redundancy in the system.

A hitherto unconsidered but potentially important source of protein for the generation of bioactive peptides is endogenous gastrointestinal proteins. Every day, copious quantities of a variety of proteins are secreted into the digestive tract sourced from the body. In fact, the total amount of endogenous protein secretion is approximately the same as the amount of protein ingested from the diet. Around 80% of the secreted endogenous proteins are digested and reabsorbed. It is interesting to speculate that the endogenous gut proteins, that are a relatively consistent supply of protein to the gut, may give rise to a consistent supply of bioactive peptides, thus supplementing the less reliable food source. This hypothesis remains to be tested.

Not only are food bioactive peptides derived from a wide variety of sources, they may also be released from food proteins by a wide variety of enzymes. This may occur during natural digestion in the digestive tract under the action of mammalian enzymes or by enzymes produced by a multitude of microbes inhabiting the human gut. Pellegrini *et al.* (1997) in comparing different proteolytic

digests of lysozyme report, for example, a highly bactericidal digest obtained from the protease clostripain produced from *Clostridium* bacteria, a member of the normal intestinal flora of humans. Alternatively, bioactive peptides may be present in the food itself, following industrial hydrolysis of protein or arising during the natural processing and ripening of the food. Microbial enzymes either in the gut or food use different cleavage sites to mammalian digestive enzymes and can give rise to different peptides. Moreover, the microbial and mammalian digestive enzymes may act synergistically.

It is known that the intestinal microflora can modify the immunomodulatory effects of foodborne bioactive peptides, and one way by which probiotic bacteria may be effective is by enhancing the formation of efficacious bioactive peptides (Rutherford-Markwick and Moughan, 2005). Gut bacteria may also secrete specific bioactive proteins and peptides.

12.3 Physiological effects of food-derived proteins and peptides

Proteins from foods and food-derived peptides have been discovered that affect a wide range of biological processes in the body. Some of the reported physiological effects of natural food-derived peptides and proteins are shown in the following list.

- modulation of gastrointestinal motility;
- stimulation of gut secretory processes;
- mineral-binding and enhanced mineral absorption;
- enhancement of gut mucus synthesis and secretion;
- inhibition of digestive enzymes;
- cholesterol lowering;
- endocrine functions;
- induction of satiety;
- antibacterial, antifungal, antiviral actions;
- anticarcinogenic action;
- immunomodulatory actions;
- antithrombotic action;
- reduced risk of cardiovascular disease;
- inhibition of ACE in the control of hypertension;
- antioxidant activity;
- osteoprotective;
- analgesic and various neuroactive (opioid) actions.

This list is unlikely to be exhaustive as hitherto unknown peptides are continually being discovered and their effects elucidated. Some peptides have more than one biological activity: β -casomorphin-7, for example, exhibits opioid and ACE inhibitory and immunomodulatory effects (Meisel, 1997). Often, these peptides are from overlapping peptide sequences in so-called strategic zones of a protein. It also

seems likely, given the potential number of food bioactive peptides and proteins, that activities are the result of the co-action of several molecules. Overall effects may need to be considered in terms of multiple molecules acting together within the context of a complex milieu, the gut contents.

12.4 Stability of bioactive peptides in the digestive tract, the potential for absorption and site of action

Bioactive peptides may act as physiological modulators locally in the digestive tract and, potentially, systemically after absorption. However, the release of peptides from proteins during digestion and their potential physiological effects have been mostly demonstrated *in vitro*. This raises some important questions: do peptides arise naturally during *in vivo* digestion? Do such peptides remain patent in the digestive tract lumen? Do the peptides act within the lumen itself or at the mucosal surface? Do peptides bind to receptors on the mucosal side of the enterocyte or the serosal side? Are peptides absorbed in the human adult and do they act systemically? Do peptides derived from food possess sufficient bioactivity to be effective at concentrations normally found in the gut lumen?

12.4.1 Do food-derived peptides arise naturally during digestion *in vivo* and do they remain patent in the gut?

A small number of studies conducted using animal models for humans or adult humans directly do demonstrate that the types of peptides found during *in vitro* hydrolysis of proteins also arise under the action of enzymes in the gastrointestinal tract and can be recovered from gut luminal contents. As bioactive peptides have been located within ileal fluid, some at least remain intact during passage through the upper digestive tract. Kelly and Coutts (1997) have discussed the important protective role milk proteins (e.g. casein) may play in the adult human, blocking the active site of proteolytic enzymes and thus limiting the hydrolysis of certain peptides. *In vitro* digestion studies suggest that some peptides released from proteins may be quite resistant to the mammalian enzymes pepsin, trypsin and chymotrypsin. Naito and Suzuki (1974) provided some evidence that casein-phosphopeptides (CPP), similar to those produced by *in vitro* (pepsin–trypsin) digestion, are found in rat small intestinal contents. The CPPs were predominantly found in the distal small intestine, demonstrating patency in the upper digestive tract. Moreover, Kasai *et al.* (1992) also working with rats, recovered CPP from faeces. Kitts *et al.* (1992) also found CPPs in the intestinal contents of rats fed casein. Meisel (1986), working with mini pigs fed casein, isolated a β -casein fragment from duodenal digesta. The fragment was shown to have opioid activity and was thus named the opioid agonist β -casomorphin 11. The author, referring to earlier unpublished work, reported no casomorphin-like peptides in gastric chyme. Meisel and Frister (1988) found CPPs in the small intestinal digesta of mini pigs fed on a diet containing bovine casein.

Evidence from humans is more sparse. Svedberg *et al.* (1985) assayed for β -casomorphin immunoreactive material in the small intestine of adult humans consuming cow's milk. β -casomorphin-7 was found in considerable amounts, but no β -casomorphin-5 was found, and only small amounts of β -casomorphin-4 or -6. Chabance *et al.* (1998) detected several peptides derived from α -S1, β - or κ -casein in the stomach chyme of adult humans after ingestion of milk or yoghurt. Smaller peptides were recovered from duodenal digesta. In working with human adults, Meisel *et al.* (2003) demonstrated the presence of CPPs in ileostomy fluid post milk and CPP ingestion. Peptide profiles were similar among the four volunteers studied.

12.4.2 Site of action within the gastrointestinal tract

Peptides once released from the parent protein are able to act directly in the oral cavity or gut lumen (e.g. antimicrobial and anticarcinogenic activity) or may exert an effect after binding to specific receptor-binding sites on the brush border membrane. Specific receptor-mediated binding of lactoferrin has been reported for intestinal epithelial cells (Hu *et al.*, 1988), and the intestinal brush border membrane appears to be the main target site in adults for the effects of exogenous opioid peptides (Guesdon *et al.*, 2006). An interaction between casomorphins and opioid receptors located on the serosal side of the intestinal epithelium with a subsequent increase in electrolyte transport has also been reported (Ben Mansour *et al.*, 1988; Bos *et al.*, 2000).

12.4.3 Are peptides absorbed in the human adult and do they act systemically?

There is good evidence that bioactive proteins and peptides can be absorbed from the immature gut of the newborn infant (Kelly and Coutts, 1997), but are they absorbed in physiologically significant quantities in the adult human?

Firstly, such absorption is possible mechanistically. The transepithelial movement of luminal peptide fragments is not completely understood, but small amounts of peptides can be absorbed intact by one or more of four general mechanisms:

1. specific transport via PepT_1 ;
2. paracellular movement across leaking tight junctions;
3. transcellular movement across the enterocyte via endocytosis/exocytosis;
4. IgA/M-cell presentation to Peyer's patches/lymphoid tissue (Moughan and Stevens, 2013).

Secondly, exogenous bioactive peptides have been detected in blood plasma, peripheral tissues and urine, showing not only that the peptides were absorbed from the digestive tract but also that they survived the action of sera enzymes and catabolism in the liver and peripheral organs. That food-derived peptides can cross the small intestine and be found in blood and tissues has been appreciated for some

time (Boullin *et al.*, 1973; Matthews, 1975; Gardner, 1975; Hemmings *et al.*, 1977). Bloch *et al.* (1988) found radioactively-labelled polypeptide fragments (produced by pepsin digestion of bovine serum albumin, MW 6000–25 000 Da) in the portal blood of the rat, after the material was infused into the jejunum. Failure to find labelled material in systemic blood pointed to rapid clearance.

Chabance *et al.* (1998) reported the presence of two long peptides (κ -caseinoglycopeptide; N-terminal peptide of α -S1-casein) in the plasma of adults consuming milk or yoghurt. The caseinoglycopeptide was detected in the plasma of all six subjects, reached a maximum concentration 40 minutes after the meal, with the concentration then remaining constant for eight hours. The caseinmacropeptide has also been found in the blood of rats after oral administration (Fosset *et al.*, 2002). ACE inhibitory peptides were found in the aortal tissues of rats after oral administration (Masuda *et al.*, 1996). Hueckel and Rogers (1970) have reported finding hydroxyproline-containing peptides in the urine of humans fed gelatin, indicating that these peptides were absorbed intact and were subsequently excreted.

Finally, indirect evidence that proteins and peptides can be absorbed in small amounts and have physiological effects peripherally comes from studies demonstrating biological effects consequent upon oral administration of polypeptides. This is discussed by Zaloga and Siddiqui (2004). In work with the rat, oral administration of three peptides with known biological effects was investigated. The peptides chosen were thyrotropin-releasing hormone (TRH, a tripeptide), luteinising hormone releasing hormone (LHRH, a decapeptide) and insulin (a 51 amino acid polypeptide). These molecules are all inactivated by cleavage at any site. Small quantities (<0.5 mg TRH and LHRH; 25 mg insulin) of the polypeptides produced distinct expected biological effects. Insulin levels in the blood increased following the enteral administration. FitzGerald *et al.* (2004) have given a good summary of animal studies and a limited number of human studies clearly demonstrating a hypotensive effect (for the hypertensive state) consequent upon the oral administration of protein hydrolysates known to contain ACE-inhibitory peptides. Satake *et al.* (2002), working with Caco-2 cells, showed that an ACE-inhibitory tripeptide (VPP) could be paracellularly transported to the basal side of the cell intact, although a significant amount of VPP was transported via the peptide transporter, PepT₁, and was hydrolysed intracellularly.

In conclusion, it seems that generally the degree of peptide absorption decreases with increasing chain length, and for some bioactive peptides there is no evidence of absorption (Teschemacher, 2003). Nevertheless, the weight of evidence would suggest that some food-derived peptides and proteins are absorbed but to differing degrees.

12.4.4 Are bioactive peptides from food sufficiently physiologically active to be effective at concentrations normally found in the gut lumen?

The affinities of food protein-derived bioactive peptides to cellular receptors appear to be weak compared to endogenous and some synthetic (drug) peptides.

However, the intestinal concentrations of the dietary peptides are, at least in some cases, relatively high such that the exogenous peptides may bring about physiologically significant effects (Pellegrini, 2003; Teschemacher, 2003; Meisel, 2005). Also, different peptides found in the lumen of the digestive tract may act synergistically.

12.5 Antimicrobial effects

12.5.1 α - and κ -casein

Over 20 peptides with anti-microbial activity have been identified following the hydrolysis of casein with digestive proteases (Benkerroum, 2010). Caseicins and isracidin are derived from a chymosin digest of α_{s1} -casein, and both exhibit antimicrobial activities against a range of micro-organisms including staphylococci and streptococci (Clare and Swaisgood, 2000). Interestingly, studies utilising isracidin have shown it to be significantly more efficacious as an antimicrobial agent *in vivo* than *in vitro*. *In vivo* studies using isracidin have shown it to have both therapeutic and prophylactic effects when used at levels similar to regular antibiotic therapy; protecting mice against *Staphylococcus aureus* and *Candida albicans*; and sheep and cows against mastitis (Clare and Swaisgood, 2000; Benkerroum 2010).

In a recent study, Hayes *et al.* (2006) demonstrated that both isracidin and two peptides derived from casein (caseicin A and B) following fermentation with *Lactobacillus acidophilus* DPC6026 had antimicrobial activity against emerging pathogens such as *Escherichia coli* 0157:H7 and *E. sakazakii*, which causes neonatal meningitis. While the use of fermentation to generate bioactive peptides is likely to result in a different array of peptides with different actions and specificities to those generated by digestive protease activity, the length of the digestion or ripening process determines whether or not bioactive peptides will be present in the final product. Cheese extracts with short fermentation times have been shown to contain antimicrobial peptides while those from cheeses with either long ripening times or significant levels of proteolysis did not (Rizzello *et al.*, 2005). Loss of bioactive peptides following extensive proteolysis is not restricted to fermented products. Lopez-Exposito *et al.* (2007) have shown that digestion with pepsin can result in partial or complete loss of bioactivity of ovine α_{s2} -casein (longer than 30 minutes) and bovine κ -casein (longer than 2 hours), as does, sequential digestion of casein utilising digestive enzymes (Lopez-Exposito *et al.*, 2006). Interestingly, it appears that the formation of other bioactive peptides such as ACE-inhibitory peptides relies on longer periods of hydrolysis. ACE-inhibitory peptides are frequently found in higher concentrations in aged cheeses, with the optimum ripening length highly dependent on the type of cheese (reviewed in Korhonen, 2009).

Bovine α_{s2} -casein is also a source of at least five antimicrobial peptides including casocidin-I which has been produced and purified via a complicated process. With activity against both Gram-negative and Gram-positive bacteria as

well as yeasts, casocidin-I has been patented for use as a treatment for dermatitis, mucosal infections and gastroenteritis (Forssmann *et al.*, 2003). Of the other bovine α_{s2} -casein-derived peptides, three (f(181–207), f(175–207) and f(164–207)) have been shown to inhibit growth of sensitive Gram-positive bacteria to a similar extent as nisin and lactoferricin B (McCann *et al.*, 2005), prompting suggestion of their use as food-grade preservatives.

κ -casein is another source of bioactive peptides with potential in the health, food safety and food preservation areas. Caseinomacropeptide (CMP, f(106–169)), also known as glycomacropeptide (GMP) is an anionic peptide formed from κ -casein during either cheese manufacture (action of chymosin) or digestion. In addition to being phosphorylated, CMP bears a variety of oligosaccharide structures that are thought to bind to pathogenic bacteria thus preventing the bacteria from binding to sites on the mucosal membrane.

CMP has been shown to prevent gastrointestinal bacterial infections in several animal challenge trials (Isoda *et al.*, 1999) and to inhibit haemagglutination of four human influenza virus strains (Kawasaki *et al.*, 1993), *Mycoplasma gallisepticum* (Glasgow and Hill, 1980) and *M. pneumoniae* (Loomes *et al.*, 1984). However, more recent work has focused on the potential of CMP in the area of oral health. Researchers suggested that CMP may help to reduce dental caries following *in vitro* studies demonstrating that CMP was able to prevent cariogenic bacterial adhesion (Schupbach *et al.*, 1996). In this case, CMP was thought to promote the growth of *Actinomyces viscosus* over the more cariogenic *Streptococcus mutans* and *S. sanguis*, thus effectively altering the bacterial populations in the dental plaque. Malkoski *et al.* (2001) showed that CMP has activity against *E. coli* and *Porphyromonas gingivalis* as well as *S. mutans*. They also demonstrated that the only active form of CMP was that which is phosphorylated but not glycosylated, a form which they have named kappacin. Phosphorylation of the serine residue at position 149 has been shown to be essential for antimicrobial activity of kappacin, as has the presence of the aspartic acid residue at position 148 (Malkoski *et al.*, 2001; Dashper *et al.*, 2005). In addition, the binding of either zinc or calcium (naturally high in milk) to the peptide has also been shown to enhance its efficacy at neutral pH (Dashper *et al.*, 2005), purportedly by causing a conformational change. Kappacin is currently being tested in two licensed products (Kappacin™ and KappaZinc™, Cooperative Research Centre for Oral Health Science, Australia) for its ability to inhibit the growth of oral bacteria.

12.5.2 Whey proteins

Previously less well studied, but now receiving considerable attention, are the peptides derived from α -lactalbumin (α -La) and β -lactoglobulin (β -Lg). Following fermentation or treatment with digestive enzymes, bovine α -La releases a range of bioactive peptides (dependent on the method of proteolysis) with a plethora of reported activities including antimicrobial and antiviral properties against *E. coli*, *Bacillus subtilis* and herpes simplex virus type 1 (Kamau *et al.*, 2010). Tryptic digestion of β -Lg also results in the production of antimicrobial

peptides against Gram-positive bacteria (Hernández-Ledesma *et al.*, 2008). However, issues exist with the allergenicity of peptides derived from milk, particularly those from β -Lg, and this, along with the fact that the peptides have only moderate activity against a narrow range of bacteria, means that these peptides have yet to find a useful application (Benkerroum, 2010).

Lactoferrin (Lf), a naturally occurring iron-binding glycoprotein found in colostrum and milk, has been ascribed a variety of biological functions including being an important antimicrobial component for protecting the host against microbial infections (Baker *et al.*, 2002). Lf has the ability to sequester iron, and at least part of its function may be directly due to this ability. Several studies have demonstrated that Lf is able to enhance bacterial susceptibility and hence lower the required therapeutic concentrations of several antibiotics such as vancomycin (Leitch and Willcox, 1999), penicillin (Diarra *et al.*, 2002) and cephalosporin (Miyazaki *et al.*, 1991 as cited by Kanwar *et al.*, 2009) and it has been suggested that this can be attributed to the iron-binding ability of Lf (Kanwar *et al.*, 2009).

However, the antimicrobial mechanism of Lf appears more complex than simply iron binding. Lf is able to exhibit non-iron-dependent bactericidal activity by binding to Gram-negative species leading to changes in membrane permeability and metabolism. Peptides derived and isolated from Lf (e.g. lactoferricin, lactoferrampin) have been shown to have a highly potent antimicrobial activity (100–1000 times higher than intact Lf) (Bellamy *et al.*, 1992) against a range of both Gram-negative and Gram-positive bacteria and certain yeast, fungi and viruses (Bellamy *et al.*, 1994; Ellison, 1994; Jones *et al.*, 1994; Tomita *et al.*, 1994; Shin *et al.*, 1998; Orsi, 2004). Nuclear magnetic resonance (NMR) studies have shown that the pepsin-liberated peptides from Lf, which carry a net positive charge, adopt an amphipathic structure which, similarly to cationic peptides, allows them to disrupt the cell membrane and enter the cell (Schibli and Vogel, 2000). Therefore peptides such as lactoferricin kill bacteria by increasing membrane permeability (Orsi, 2004). Evidence suggests that lactoferricin is generated following digestion of Lf in the human stomach (Kuwata *et al.*, 1998) and gastrointestinal contents of rats (Tomita *et al.*, 1994). However, the *in vivo* bactericidal properties of lactoferricin have been questioned, as both the addition of 5% cow's milk or adding increasing concentrations of mucin to the assay reduced the antimicrobial effects (Jones *et al.*, 1994).

12.5.3 Other food sources

It is not only milk proteins and peptides that have antimicrobial activity. Egg yolk digests have been shown, for example, to prevent bacterial infection in chickens (Kassaify and Milne, 2004), reportedly by having an anti-adhesive action (Kassaify *et al.*, 2005). Studies investigating other possible mechanisms of action have been carried out in mice administered egg yolk low-lipid peptic digests, and these showed that the digest enhances mucosal immune responses by increasing IgA⁺ cells and increasing interleukin (IL)-6 secretion by small intestinal epithelial cells

(Nelson *et al.*, 2007). Lysozyme and peptides from egg white lysozyme have been shown to have antimicrobial activity, some of which is due to activity which is not enzymatic in nature (Mine *et al.*, 2004).

12.6 Effects on immune function

Bovine milk contains multiple peptide fractions that can affect immune function, leading, amongst other benefits, to improved resistance to infection. Most of these peptides are derived from the hydrolysis of the major milk proteins through proteolysis, either by using digestive enzymes or by fermentation, although some are naturally occurring and require little or no modification to become active (Stoeck *et al.*, 1989; Guimont *et al.*, 1997). The specific effects seen include modulating human lymphocyte proliferation, maturation of T cells and natural killer (NK) cells, enhancement of phagocytic activity and increased antibody production.

Proline-rich polypeptide, also known as colostrinin (CLN), occurs naturally in milk, and is known for its immunoregulatory properties. CLN contains about 22% proline, and originally it was suggested that this high proline content may confer significant resistance to digestion; however, a more recent study has demonstrated that CLN consists of at least 32 different peptides (Kruzel *et al.*, 2001). CLN appears to have a wide variety of functions including immunomodulation (B and T cell activation), promoting cell proliferation, improving wound healing and having antioxidant, antihypertension and neuroprotective activities (summarised in Kanwar *et al.*, 2009).

Synthetic peptides corresponding to those generated from CLN following digestion with chymotrypsin have been shown to stimulate antibody responses to sheep red blood cells in mice (Janusz *et al.*, 1987). In an autoimmune mouse model, CLN was shown to reduce autoantibody production (Hraba *et al.*, 1986). CLN has been shown to induce cytokine production by murine macrophages (Julius *et al.*, 1988) and human whole blood cell cultures (Zablocka *et al.*, 2001) as well as promoting the growth and differentiation of resting B lymphocytes in mice (Julius *et al.*, 1988). CLN is able to reduce intracellular levels of reactive oxygen species (Boldogh *et al.*, 2003), and it is via this mechanism that CLN has been postulated as a useful tool to aid in the prevention of age-related changes to the immune system, central nervous system and skin (Kanwar *et al.*, 2009).

Recent *in vivo* and *in vitro* studies have shown that CLN appears to have neuroprotective effects, reducing aggregation of amyloid protein, resulting in enhanced cognitive function and memory retention (Stewart and Banks, 2006), and hence it may be useful in treating or slowing the progression of Alzheimer's disease. Studies in this area have advanced to human testing with promising results (Leszek *et al.*, 1999, 2002; Bilikiewicz and Gaus, 2004). The most recent of these studies (Bilikiewicz and Gaus, 2004) has demonstrated the efficacy of CLN (trademarked as Colostrinin™) in stabilising cognitive function, particularly in patients in the early stages of the disease.

A number of peptides derived from casein have been found to be immunostimulatory, with each of the caseins producing different bioactives and hence different effects depending on the enzymatic processes used to generate them (reviewed by Gill *et al.*, 2000). For example, casein digested with both pepsin and trypsin resulted in peptides which were immunomodulatory toward human lymphocytes *in vitro*, whereas casein hydrolysed by the proteolytic enzymes from *Lactobacillus GG* var *casei* prior to the pepsin/trypsin treatment were immunosuppressive (Sutas *et al.*, 1996a, b). Pancreatin and trypsin digests of both α_1 -casein and β -casein were shown to inhibit proliferation of both murine spleen cells and rabbit Peyer's patch cells (Otani and Hata, 1995) whereas digests prepared using pepsin and chymotrypsin had no effect. Lorenzen and Meisel (2005) have shown that by digesting yoghurt milk with trypsin prior to fermentation a number of bioactive peptides are produced, whereas fermentation alone results in minimal proteolysis.

A wide range of immunomodulatory actions has been described for specific peptides derived from α_1 -casein, including suppression of *in vitro* mitogen-stimulated human peripheral blood mononuclear cell proliferation (Kayser and Meisel, 1996), promotion of immunoglobulin formation (Hata *et al.*, 1998), enhancement of phagocytosis *in vitro* (Jollès *et al.*, 1992; Schlimme and Meisel, 1995; Meisel, 1997) and enhancement of lymphocyte proliferative responses, NK cell activity and neutrophil locomotion (Migliore-Samour and Jollès, 1988; Elitsur and Luk, 1991).

Peptides derived from β -casein have been shown to have concentration-dependent effects on mitogen-induced proliferation of human lymphocytes, suppressing proliferation when present at low concentrations and enhancing it at higher concentrations (Kayser and Meisel, 1996). Other studies have shown that peptides from β -casein stimulate proliferation of rat lymphocytes in the absence of mitogens (Meisel, 1997); while yet others have shown that following digestion of β -casein with pancreatin and trypsin, there is inhibition (*in vitro*) of mouse spleen lymphocyte proliferation (Otani and Hata, 1995), whilst the peptide comprising residues 1–25 from β -casein which is rich in phosphoserine residues stimulated proliferation of mouse splenocytes (Hata *et al.*, 1998). These opposing results could be due to several reasons including: the concentration-dependent effect demonstrated by Kayser and Meisel (1996) mentioned above, differences in peptides generated as a result of protease choice, or species specific differences in the animal model used. β -casein derived peptides have also been shown to promote antibody formation, enhance phagocytic activity of murine peritoneal cells and enhance antigen-dependent T cell proliferation (Jollès and Migliore-Samour, 1986; Jollès *et al.*, 1988; Migliore-Samour and Jollès, 1988).

In vitro studies with CMP derived from κ -casein, have shown it to be a potent inhibitor of LPS- and phytohaemagglutinin (PHA)-induced proliferation of murine splenic lymphocytes (Otani *et al.*, 1995), with the level of inhibition being proportional to the carbohydrate content (as cited by Moller *et al.*, 2008). A more recent study by Otani *et al.* (2005) demonstrated that the inhibition of PHA-induced proliferation was dependent on the strain of mouse from which the spleen

cells were sourced, possibly due to the differing ability of CD4⁺ T cells from the strains tested to bind CMP.

CMP also inhibited proliferation of rabbit Peyer's patch cells, as well as suppressing antibody production by murine spleen cell cultures (Otani *et al.*, 1995). In contrast, *in vitro* studies by other workers have shown that specific peptides generated by digestion of κ -casein are able to enhance cellular proliferation (Kayser and Meisel, 1996; Meisel, 1997). For example Sutas *et al.* (1996b) found that peptides from a pepsin–trypsin digest of κ -casein significantly enhanced human lymphocyte proliferation. Specific peptides from κ -casein digests have also been shown to promote antibody formation and enhance phagocytic activity of murine and human macrophages (Jollès and Migliore-Samour, 1986; Jollès *et al.*, 1988).

A number of the bioactive peptides from casein appear to be multifunctional. β -casomorphins have been shown to protect mice against *Klebsiella pneumoniae* infection, an effect which was suppressed by the administration of an opioid antagonist, hence suggesting the effect is mediated via an opioid receptor (Parker *et al.*, 1984; Elitsur and Luk, 1991). β -casein-derived ACE inhibitor peptides have also been shown to stimulate phagocytosis (Jollès *et al.*, 1992; Schlimme and Meisel, 1995).

Hydrolysis of the whey proteins has led to preparations which are capable of modulating the immune system whilst, in some cases, no activity or inhibitory activity is seen with the intact protein (Mercier *et al.*, 2004). A diet of hydrolysed α -lactalbumin was able to modulate T and B lymphocyte function (Bounous and Kongshavn, 1985) and enhance antibody responses to systemic antigen stimulation (Bounous and Kongshavn, 1982). Several immunomodulatory peptides derived from α -lactalbumin have been identified and studied using both the natural and synthetic forms of the peptide. These peptides have been shown to exhibit a range of functions including stimulation of phagocytosis and respiratory burst, protection of mice against *K. pneumoniae* infection and enhancement of human peripheral blood lymphocyte proliferation (reviewed in Kamau *et al.*, 2010). *In vitro* studies with Lf have shown that the intact protein can inhibit lymphocyte proliferation (Mercier *et al.*, 2004), while the peptide lactoferricin (residues 17–41 of LF) can suppress IL-6 production by a human monocyte cell line following stimulation with LPS (Mattsby-Baltzer *et al.*, 1996), stimulate the release of IL-8 from human polymorphonuclear leucocytes (Shinoda *et al.*, 1996) and promote the phagocytic activity of human neutrophils (Miyauchi *et al.*, 1998). Roos *et al.* (cited by Moller *et al.*, 2008) carried out a double-blind placebo-controlled study in 95 elderly subjects investigating the effects of dietary supplementation with Lf on immune response. Subjects were given 0.9 g Lf prior to and following an influenza vaccination which resulted in an increased percentage of granulocytes (of total leukocytes) in the Lf-supplemented group both on day 0 prior to vaccination and also seven days after vaccination. In addition, the phagocytic activity of the granulocytes from the Lf-supplemented group was higher one day after vaccination.

Lf and Lf-derived peptides have both been demonstrated to have antitumour

activity using cell lines *in vitro* (Yang *et al.*, 2002; Yamada *et al.*, 2008) and *in vivo* models (Bezault *et al.*, 1994; Wang *et al.*, 2000). A study by Kanwar *et al.* (2008) demonstrated that bovine Lf fed at least two weeks prior to chemotherapy was able to eradicate tumours from mice, although the Lf had to be saturated with iron in order to be effective.

Immunostimulating peptides from sources other than milk also exist. For example soymetide, a peptide derived from soyabean protein, stimulates the phagocytic activity of human polymorphonuclear leukocytes *in vitro* (Yoshikawa *et al.*, 2000), and oral consumption of a fermented fish protein concentrate has been shown to enhance gut-associated non-specific immunity in mice (Duarte *et al.*, 2006). This study showed that consumption of the fermented fish protein concentrate led to enhancement of peritoneal macrophage phagocytosis and increasing IL-4, IL-6, IL-10, interferon (IFN)- γ and tumour necrosis factor (TNF)- α levels, but interestingly an inflammatory response was not elicited, despite increases in some pro-inflammatory cytokines (Duarte *et al.*, 2006). The protein or peptides responsible for this effect were not identified.

A recent focus in the area of bioactive peptides and proteins has been on those derived from pulses. Studies have shown that lectins may have a key role in preventing certain cancers, with lectins from different sources potentially acting on different types of cancers and by different mechanisms. Studies including some in animal models have shown that lectins from mistletoe, lentils and *Pisum sativum* are able to decrease the mitotic activity, tumour size and tumourigenicity in a diverse range of cancers including non-Hodgkins lymphoma, human hepatoma and skin carcinomas (Roy *et al.*, 2010). In addition, mistletoe lectins are able to improve the efficacy of chemotherapy by inducing cytokine activity in mononuclear cells (Pryme *et al.*, 2006). Such a product is currently available in Europe and is purportedly being used as an adjunct to chemotherapy.

Pulse-derived denatured protease inhibitors, particularly soyabean Bowman-Birk inhibitor (BBI), have also been reported to suppress colon, oesophageal, liver, lung and oral carcinogenesis in animal models (Moy and Billings, 1994). BBIs appear to be resistant to digestion and are transported to tissues within the host, as studies have shown that following consumption of BBI the activity can be detected in a variety of tissues and organs in the body (Moy and Billings, 1994).

12.7 Effects on inflammation

Much of the work on anti-inflammatory bioactive peptides has focused on their potential for the maintenance of gut integrity in inflammatory diseases, namely to ameliorate inflammatory bowel disease (IBD), a term which encompasses a complex group of diseases, of which Crohn's disease and ulcerative colitis are the most prevalent. The disease is thought to result from an inappropriate immune response to intestinal microflora. The quality of life of patients suffering from IBD is severely affected due to its chronic and relapsing nature. In addition, current treatments which include immunosuppressive agents have significant side effects;

therefore new less deleterious treatments are being sought. Evidence so far indicates that at least two milk-derived bioactive peptides, transforming growth factor (TGF)- β and CMP, may be useful in treating IBD.

TGF- β , found in both milk and colostrum, has been implicated in epithelial cell growth and differentiation and has been shown to aid in the repair of injured tissue (Donnet-Hughes *et al.*, 2000). Favourable results have been found in trials investigating the effects of diets containing TGF- β on various forms of IBD, including reduced intestinal inflammation (Beattie *et al.*, 1994; Ozawa *et al.*, 2009), reduced leukocytes and acute phase reactants in the bloodstream (Schriffin *et al.*, 2005), decreased expression of IFN- γ (Beattie *et al.*, 1994; Fell *et al.*, 2000) and induction of remission and promotion of mucosal healing (Beattie *et al.*, 1994; Fell *et al.*, 2000; Donnet-Hughes *et al.*, 2000). Both systemic administration and induction of TGF- β have been shown to have protective effects against induced colitis, and administration of anti-TGF- β antibodies appears to ablate this effect (Neurath *et al.*, 1996). These results demonstrate the important role that TGF- β has in preventing the development and manifestations of IBD in animal models (Sanchez de Medina *et al.*, 2010).

Denatured protease inhibitors from pulses also have the potential to be used as anti-inflammatory agents, with consumption of soyabean BBI leading to a decrease in inflammation of the colon in an induced ulcerative colitis mouse model compared to a control diet (Ware *et al.*, 1999).

In experimental animal models of colitis and ileitis, oral administration of CMP was shown to have significant anti-inflammatory effects (Daddaoua *et al.*, 2005; Requena *et al.*, 2008). One trial suggests that the efficacy is similar to that of sulfasalazine, a drug commonly used in the treatment of IBD (Daddaoua *et al.*, 2005).

There has been considerable work demonstrating the ability of bioactive peptides from a variety of protein sources such as bonito, wheat germ, gelatin and β -casein to act as angiotensin I-converting enzyme inhibitors and hence lower blood pressure (Moller *et al.*, 2008). Several of these peptides have also been studied to determine if they also have an anti-inflammatory role. Results appear to be mixed and more work is required to determine if food-derived ACE inhibitors are able to affect inflammatory indices (Owusu-Apenten, 2010).

12.8 Mechanisms of action

Milk is well recognised as an important nutritional source for the neonate and it appears likely that bioactive milk peptides play additional roles by assisting in the development of the gastrointestinal tract, aiding in the prevention of hypersensitivity to nutrients, and protection of the infant from bacterial and viral enteric and respiratory infections.

Debate continues on the mechanism of action of antimicrobial peptides, but most are believed to act by adopting amphipathic structures enabling interaction via electrostatic bonding and hence disruption of the bacterial cell membrane at

least to some extent (Dashper *et al.*, 2007). Whether the disruption is transient, thus preserving the cell's integrity but resulting in changes in membrane permeability and/or energy generation, or total, resulting in cell disintegration, is currently unknown. Although the validity of using membrane models to elucidate mechanisms of action has been questioned, studies using synthetic liposomes indicate that kappacin may work by increasing membrane permeability and hence causing bacterial cell death by disrupting the membrane structure (Dasper *et al.*, 2005).

The observed differences in antimicrobial efficiency of isracidin *in vitro* and *in vivo*, along with results demonstrating that isracidin stimulates both cellular and humoral immune responses (Lahov and Regelson, 1996), suggest that it is working indirectly as an immunomodulatory peptide improving resistance to infection rather than by directly killing the bacteria (Benkeroum, 2010).

The antimicrobial effects displayed by Lf and lactoferricin appear to be due to multiple mechanisms, including iron-binding capacity, inhibiting attachment and invasion, acting as a serine protease and cleaving colonisation factors, amplifying apoptotic signals in infected cells, increasing cell membrane permeability and promoting bactericidal activity of granulocytes (Orsi, 2004). Several studies have shown that Lf is able to indirectly confer antimicrobial activity by affecting the immune system, and significantly enhancing the killing rate of bacteria such as *E. coli* and *S. aureus* (Kanwar *et al.*, 2009).

The mechanisms of action through which bioactive peptides elicit their effects on the immune system are largely unknown. A number of hypotheses have been suggested, including stimulation of differentiation, maturation and proliferation of different cell types such as T cells and NK cells; and it has been suggested that milk-derived opioid peptides may act on lymphocytes via the opiate receptor, thereby affecting the reactivity of the cells (Meisel, 1997). It has also been suggested that certain structural motifs are important for activity, such as the presence of an arginine residue in the N- or C-terminal region of the peptide, the presence of tryptophan-rich sequences or the presence of phosphoserine residues in CPPs (Haque *et al.*, 2009). Peptides high in glutamine are also thought to exert non-specific immunostimulation due to their ability to substitute for free glutamine, which is required for lymphocyte proliferation (Haque *et al.*, 2009). However, it is known that certain immunomodulatory peptides, such as those derived from tryptic digests of rice and soyabean proteins, act by stimulating reactive oxygen species which in turn leads to activation of the non-specific immune system (Kitts and Weiller, 2003), and there is increasing evidence that bioactive peptides are able to specifically modulate cell viability, triggering apoptosis, particularly of malignant cells while leaving normal cells unaffected (Hartmann and Meisel, 2007).

The iron-binding ability of Lf has been proposed as one mechanism by which it exhibits antitumour properties as rapidly dividing cells require iron for growth. In addition, iron-binding may lead to a reduction in oxidative stress by helping to control the balance between production and elimination of reactive oxygen species (Marnett, 2000; Jain *et al.*, 2006; Nishikawa *et al.*, 2007). This is important as a number of degenerative processes and diseases such as cancer, ageing, and atherosclerosis have been attributed to oxidative stress (Kanwar *et al.*, 2009).

Proposed mechanisms of action for the anticancer effects of pulse lectins vary, although it has been suggested that they are able to bind to cell membranes or receptors and cause cytotoxicity and apoptosis and, since some lectins such as *Pisum sativum* agglutinin have been shown to have a high affinity for tumour cells, targeted death of tumour cells seems possible. Other proposed mechanisms include agglutination, activation of protein kinases, inducing neutrophil and lymphocyte migration, activation of mononuclear cells and spleen lymphocytes, and improving host immunocompetence (Roy *et al.*, 2010); however, a significant amount of research is still required in this area. It is known, however, that lectins are immunomodulatory and are able to enhance several immune parameters including lymphocyte proliferation, NK cells activity, cytokine regulation and antibody production (Hartmann and Meisel, 2007), thus presenting another potential mode of action.

The action of TGF- β in preventing the induction of IBD appears to be by regulating suppressive cells; inhibiting activation of T (primarily Th1) and B cells by preventing the expression of IFN- γ , which, in turn, protects the epithelial monolayer from the effect of TNF which has permeability-enhancing effects (Donnet-Hughes *et al.*, 2000; Sanchez de Medina *et al.*, 2010), although further work is required. A number of studies investigating the immunomodulatory effects of CMP have led to the conclusion that it is able to modulate inflammatory responses via the activation of macrophages, favouring differentiation of Treg cells and hampering Th1 cell activation (Sanchez de Medina *et al.*, 2010). Work by Daddaoua *et al.* (2005) and Requena *et al.* (2008) has shown that in IBD, CMP decreases the expression of a number of cytokines including IL-1 β , IL-1 α , TNF and IL-17, leading the authors to conclude that the mechanism of action of CMP is through the actions of Th17 cells, macrophages and T cells but not Th1 cells. In addition, microarray studies indicate that CMP is able to downregulate IL-1 β and IL-6 genes (Sanchez de Medina *et al.*, 2010), and there is increasing evidence that IL-6 is a key cytokine involved in the chronic inflammatory process of colitis.

The mechanism of action of protease inhibitors in suppressing carcinogenesis and inflammation is not understood. Several hypotheses have been suggested, including inhibition of cellular enzymes (Moy and Billings, 1994), altering proteolytic and hydrolysing activities and altering oncogene expression (Roy *et al.*, 2010). Therefore significant research is clearly still required in this area.

12.9 Applications in human health and clinical nutrition

Many possible opportunities exist for the use of bioactive peptides and proteins as functional ingredients aimed at improving human health. Products are appearing that are aimed at numerous target groups, not just for health-conscious individuals, but also for individuals with specific conditions such as IBD, prevention of graft vs host disease, regulation of inflammation in autoimmune disorders and hypertension. Other suggested options for the use of bioactives include their use in the treatment of diarrhoea, cancer prevention and treatment, dental health applica-

tions, oxidative stress, immunodeficiency and prevention of infection from bacteria, parasites and viruses, etc. (Haque *et al.*, 2009). A number of bioactive proteins and peptides are now commercially available, either as ingredients for food and non-food matrices (e.g. toothpaste) or functional food products which carry associated health claims (Hartmann and Meisel, 2007).

Theoretically, antimicrobials have the potential for use in the medical field as antibiotics, however, these peptides, some of which were discovered as early as the 1930s (Jones and Sims, 1930), have generally been found to be less effective than their regular counterparts. Since their production would also be technically difficult and comparatively more expensive, commercial interest in this application has diminished.

Aside from use as antibiotics, a variety of alternative uses for antimicrobial peptides have been suggested, such as using casocidin-I in infant formulae (Zucht *et al.*, 1995). Using specifically fermented casein-containing antimicrobial peptides such as casecidin A and B (Hayes *et al.*, 2006) and kappacin (Benkerroum, 2010) as a food ingredient and/or preservative would confer a natural protection against pathogenic bacteria (Forssman *et al.*, 2001). Sourcing food preservatives from bovine milk is potentially advantageous as consumption of dairy products has long been considered safe. In addition, it is likely that at least some of the bioactive peptides from bovine milk are already present in dairy products and therefore have been consumed albeit in lower quantities than what is optimal, perhaps for centuries.

The latest consumer trends towards the use of more natural ingredients and functional foods to promote better health is now encouraging interest in the use of antimicrobial peptides as both food-grade preservatives and health-promoting food products and supplements (Benkerroum, 2010). Interest in the use of antimicrobial peptides may increase as the list of antibiotic resistant bacteria continues to grow.

There is evidence to suggest that bioactive peptide activity is dependent on its matrix, with factors such as pH and ingredient composition impacting to varying degrees. For example, purified bioactive peptides from milk frequently show activity individually but, when mixed together, no activity is seen, perhaps as a mechanism to prevent over-stimulation of the immune system. The pH can also impact greatly on the activity of bioactive peptides, with antibacterial peptides such as kappacin being lost or reduced at neutral pH (Malkoski *et al.*, 2001; Dashper *et al.*, 2005, 2007). Thus optimisation of the ingredient matrix could be one key for the future potential use of these bioactive ingredients. In addition, since cost of preparation is also a negative factor towards the use of these antimicrobial peptides, the use of crude preparations has been suggested (Benkerroum, 2010) and, since matrix is an important factor, a crude preparation may in fact provide a more favourable environment for bioactivity than a highly purified one. In addition, a crude preparation may contain further bioactive peptides which may act additively (Zucht *et al.*, 1995) or synergistically, possibly leading to better results in a significantly more cost effective manner than a highly purified preparation.

The fact that there are casein-based, lactose-free, TGF- β -enriched formulas

currently available on the market indicates that there is a role for bioactive peptides as components of enteral diets used as therapy for IBD. Although the formulas have been shown to be less effective than treatment with corticosteroids, and they are more effective in paediatric patients than in adults (Fell, 2005), they nonetheless have a place in the market given their lack of side effects compared to corticosteroid use.

Mechanisms for enhancing bioavailability of bioactive peptides are also important, and investigations into the use of mechanisms to protect bioactive peptides such as microencapsulation, lipid-based carriers, or through mucosal delivery are in progress. Further development of these technologies should ensure that bioactive peptides and proteins are more bioavailable and therefore potentially more efficacious.

There are several issues which must be taken into account when considering the usefulness and applications of bioactive food ingredients. One is that much of the work investigating the beneficial effects of proteins and peptides on health, and particularly on immune function, has been carried out *in vitro*, with only limited studies demonstrating effects in humans or in animal models. Therefore, a considerable amount of work still needs to be done to demonstrate efficacy *in vivo*.

Another issue associated with immuno-enhancing functional foods and nutraceuticals is the potential for consumption to lead to over-stimulation of the immune system, which could lead to negative effects such as chronic inflammation, allergy and autoimmune disorders. Some bioactive peptides and proteins from milk in particular are immunomodulatory as opposed to being purely stimulatory, only exhibiting their action when they are in their isolated form, with lesser activity seen when they are part of a product containing other bioactive milk proteins. This suggests that milk is perhaps already naturally balanced to prevent over-stimulation of the immune system.

Yet another issue that has plagued work on bioactive peptides and proteins is that while they may exhibit activity *in vitro*, frequently they are hydrolysed during normal digestive processes resulting in loss of bioactivity *in vivo*. In addition, for a bioactive peptide or protein to elicit a beneficial effect, it will need to be present at a certain level, and quantifying the amount present, particularly when the food protein is a minor part of a complex mix, is extremely challenging. Mass spectrometry techniques are increasingly being used to overcome this difficulty (del Mar Contereras *et al.*, 2008) and to quantify levels of bioactive peptides in both functional food products and biological fluids. Recently, Sato *et al.* (2008) have described a process by which food-derived bioactive peptides can be isolated, identified and quantified from blood following consumption of relevant foods or enzymatic hydrolysates. These processes and techniques will allow identification of bioactive peptides produced and absorbed from intact food proteins during digestion as well as those prepared and fed as hydrolysates which are able to resist (partially or completely) hydrolysis in the gut and determine if the levels are such that the peptides will be efficacious.

Even though many of the foods from which bioactive peptides and proteins are derived have generally recognised as safe (GRAS) status it is still important that

safety elements of new functional foods and ingredients are taken into consideration, particularly in products where ingredients have been enriched. Since foods are consumed over long periods of time, it is imperative that the safety of long-term ingestion be considered in terms of toxicity, potential allergenicity and cytotoxicity. It is also essential that scientific studies have shown that consumption of the products leads to promotion of health in a measurable way, and it is perhaps in these two areas of safety and efficacy where data are most lacking. Only once this has been achieved will bioactive peptides and proteins come to the fore in enhancing human health.

12.10 References

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13

Gangliosides, immunity, infection and inflammation

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Abstract: Gangliosides are negatively charged glycosphingolipids widely distributed in most vertebrate tissues and fluids. Human milk gangliosides might modulate physiological processes that take place in the early development of infants. This chapter describes the role of gangliosides as regulators of cell membrane organization and function, and in cell–cell recognition during normal differentiation or tissue targeting. As major components of membrane raft domains, dietary gangliosides may potentially influence a wide range of biological processes, including immune function, inflammation, neuronal signaling, entry of pathogens through the gut barrier and insulin resistance in metabolic disorders. Being involved in these processes, gangliosides may be critical for normal development and function, but they also might be involved in pathological processes.

Key words: gangliosides, cell–cell recognition, infection, immunity, inflammation.

13.1 Introduction

This chapter is focused on describing the potential role of dietary gangliosides in several functional outcomes, such as modulation of immune response, prevention of infection and regulation of inflammation. It begins with a description of the structure and most commonly used nomenclature for gangliosides and brief descriptions of the general distribution of gangliosides and methods used to quantify them in tissues or fluids. Next, dietary sources of gangliosides are described, mainly focusing on milk. Details of the differences in the concentration and distribution of gangliosides between bovine and human milk are included, as well as information on the composition of gangliosides in infant formulas. Then the different mechanisms of action that have been attributed to

gangliosides to explain their functional effects are described, including their role as cell surface receptors for enterotoxins, target receptors for bacterial adhesion in specific tissues and modulators of proliferation, activation and differentiation of immune cells and the production of cytokines. Their roles as receptors in cell–cell recognition, in lipid raft formation and in regulating microglia and responsiveness of signaling proteins are also mentioned. The roles of gangliosides in modulating the immune system and in preventing infection based on some of the mechanisms of action already mentioned are described. Indirect effects through modification of the intestinal microflora and also direct effects on the intestinal immune system and consequences on the systemic immune system are considered. Then recent literature describing how gangliosides might interfere with several processes regulating inflammation, such as modulation of Toll-like receptor (TLR) expression, modulation of vaso-active mediators and pro-inflammatory signal suppression, and inhibition of degradation of gut occludin tight junction protein during gut acute inflammation are covered. Finally, the existing literature related to studies using dietary gangliosides in different clinical situations is reviewed.

13.2 Definition and description of gangliosides

Gangliosides are negatively charged glycosphingolipids that consist of a hydrophobic ceramide and a hydrophilic oligosaccharide chain bearing one or more sialic acid residues in addition to a number of sugars, namely glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine (Wiegandt, 1982). Ceramide is an N-acylsphingosine in which the acyl residue is usually a saturated fatty acid with a chain length of more than 14 carbons. Svennerholm developed the classical and most frequently used nomenclature for gangliosides, which is based on two letters and one subscript number indicating differences in composition and metabolic pathway (Svennerholm, 1963). Attempts to develop more systematic approaches to naming gangliosides, such as that established by the Commission of the International Union of Biochemistry have not gained popularity, because of their complexity (Wiegandt, 1982). Figure 13.1 shows the pathway of biosynthesis of gangliosides.

Although gangliosides were initially isolated from the brain and are especially abundant in neural tissues, they are widely distributed in most vertebrate tissues and fluids. Gangliosides are usually isolated from these sources by total lipid extraction and solvent partition. Total gangliosides are usually quantified as lipid-bound sialic acid (LBSA) by colorimetric determination. To know the actual concentration of gangliosides in these tissues or fluids, it is necessary either to know the distribution of individual gangliosides and their molecular weight, or to use alternative techniques such as high-performance liquid chromatography or mass spectroscopy and nuclear magnetic resonance combined with authentic standards.

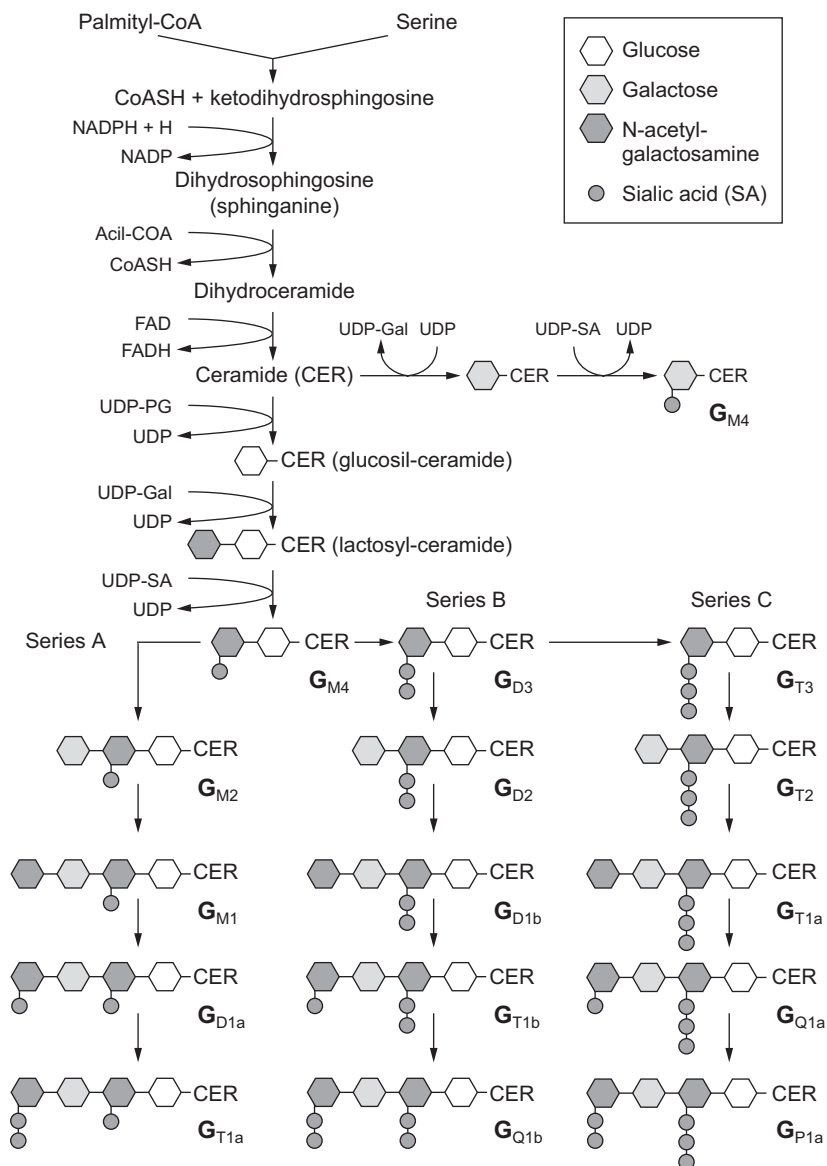


Fig. 13.1 Biosynthesis pathway for gangliosides. (a) Biosynthesis of lactosylceramide. (b) Biosynthesis of gangliosides from lactosylceramide. Source: Rueda R. (2007), 'The role of dietary gangliosides on immunity and the prevention of infection', *Br J Nutr*, 98, S68–S73.

13.3 Sources and intakes of gangliosides

There are no data about the specific content of gangliosides in foods, except for milk, but there are some data available about the content of sphingolipids in foods. The sphingolipids of mammalian tissues, lipoproteins and milk include ceramides, sphingomyelins, cerebrosides, gangliosides and sulfatides; plants, fungi and yeast have mainly cerebrosides and phosphoinositides. The total amounts of sphingolipids in food vary considerably, from a few micromoles per kilogram (fruits) to several millimoles per kilogram in rich sources such as dairy products, eggs and soybeans. With the use of the limited data available, per capita sphingolipid consumption in the USA can be estimated to be on the order of 150–180 mmol (around 115–140 g) per year, or 0.3–0.4 g/day. There is no known nutritional requirement for sphingolipids; nonetheless, they are hydrolyzed throughout the gastrointestinal tract to yield metabolites (ceramides and sphingoid bases) that are known to be used by cells to regulate growth, differentiation, apoptosis and other cellular functions. Dietary sphingolipids do not contribute much to daily energy needs of animals, nor do they appear to be ‘essential’ nutrients, although this has not yet been explored in special circumstances or disease. Nonetheless, given their potent biological activities and widespread occurrence in food, it is likely that sphingolipids can be categorized as ‘functional’ components of food (Vesper *et al.*, 1999).

From a nutritional point of view, the presence of gangliosides in milk, as well as the changes in content and individual profile that occur during lactation, could have special relevance. Human and bovine milk present a different content and distribution of gangliosides (Puente *et al.*, 1992, 1994, 1995, 1996; Rueda *et al.*, 1995, 1996a, 1996b). In human milk, G_{D3} is the most abundant ganglioside at the beginning of lactation, while at the end of this period G_{M3} is the major ganglioside (Laegreid and Otnaess, 1986; Laegreid *et al.*, 1986; Takamizawa *et al.*, 1986; Rueda *et al.*, 1995; Pan and Izumi, 1999). Table 13.1 shows the average content of total gangliosides and of G_{D3} plus G_{M3} , expressed as LBSA and actual concentration, for different periods of lactation, and for the main studies referred to above. The average value of G_{D3} plus G_{M3} in human milk, weighted according to the number of samples, would be 13.2 mg/L considering colostrum, transitional and mature human milk, or 11.1 mg/L, when only mature human milk is considered (Takamizawa *et al.*, 1986; Rueda *et al.*, 1995; Pan and Izumi, 1999).

Several authors have also studied the composition of gangliosides and other sialoglycoconjugates in infant formulas and compared it to that of human milk (Sánchez-Díaz *et al.*, 1997; Pan and Izumi, 2000). Since cow’s milk is used to manufacture infant formulas, the ganglioside distribution is similar in the two cases. Nevertheless, both the pattern and content of gangliosides in human milk and infant formulas differed markedly – G_{D3} is the main species detected in infant formulas and their total ganglioside content is significantly lower than that of human milk.

Our group has also recently described the content of total sialic acids (SAs) and gangliosides, measured as LBSA, in a series of starter infant formulas from different brands and countries and compared these results with the human milk

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Table 13.1 Average content of total gangliosides and of G_{M3} and G_{D3} (expressed as mg LBSA/L and as mg gangliosides/L) at different stages of lactation

Author	Period of lactation	<i>n</i>	Total gangliosides (mg LBSA/L)	G_{M3} (%)	G_{D3} (%)	G_{M3} (mg LBSA/L)	G_{M3} (mg LBSA/L)	G_{M3} (mg/L)	G_{D3} (mg/L)	$G_{M3} + G_{D3}$ (mg/L)
Takamizawa <i>et al.</i> (1986)	Colostrum	6	5.77	9.05	70.52	0.51	4.07	1.98	10.39	12.37
	Transitional milk	5	4.50	18.02	62.02	0.73	2.85	2.85	7.26	10.11
	Mature milk	6	4.04	60.82	20.30	2.38	0.88	9.24	2.23	11.47
Rueda <i>et al.</i> (1995)	Colostrum	4	2.59	6.38	46.20	0.17	1.13	0.65	2.89	3.54
	Transitional milk	7	5.03	13.04	36.81	0.76	2.02	2.97	5.16	8.13
	Mature milk	7	1.79	44.23	15.30	0.62	0.38	2.42	0.96	3.38
Pan and Izumi (1999)	Colostrum	16	9.82	3.13	48.19	0.31	4.73	1.19	12.07	13.25
	Transitional milk	35	9.08	26.73	33.97	2.45	3.08	9.53	7.84	17.37
	Mature milk	10	8.02	35.76	24.71	2.88	1.98	11.18	5.05	16.23

Molecular weights: sialic acid = 309; milk G_{M3} = 1198.5; milk G_{D3} = 1577. Colostrum: 1–5 days; transitional milk: 6–17 days; mature milk: more than 18 days.

Source: Rueda (2007), 'The role of dietary gangliosides on immunity and the prevention of infection', *Br J Nutr*, 98, S68–S73.

data from the literature (Martin *et al.*, 2008). Data for SA content of the different formulas, measured as N-acetylneuraminic acid (Neu5Ac), varied from 11 to 35 mg/100 mL (mean 19.4 ± 0.6 mg/100 mL). Different starter formulas from the same brand were similar, except for those claiming the exogenous addition of SAs, which were richer than the rest. Even in these fortified formulas, the level of SAs remained very low when compared to human milk content, which varies from 120–150 mg/100 mL in colostrum to 30–45 mg/100 mL in mature milk. In agreement with our data, formulas have been reported to contain less than 25% of mature milk SA content (Wang *et al.*, 2001). LBSA content of the analyzed commercial formulas was on average 0.24 ± 0.10 mg/100 mL, ranging from 0.09–0.53 mg/100 mL. As mentioned above, human milk LBSA content is highly variable. Reported data ranged from 0.2–1 mg LBSA/100 mL (Takamizawa *et al.*, 1986; Rueda *et al.*, 1995; Pan and Izumi, 1999; Rueda, 2007). The content is higher in colostrum and decreases as lactation progresses. Therefore, bottle-fed infants receive a significantly lower amount of LBSA than breast-fed babies during the first month of life. According to these data, total SA and LBSA contents in the commercial formulas remain far from those of human milk. Considering that both types of molecules might play an important role in functional development in the newborn, the supplementation of infant formulas with those molecules could be influential in neonatal physiology.

The role of gangliosides in human milk has not been well established, but they might participate in physiological processes that take place in the early development of infants (Rueda and Gil, 1998; Rueda *et al.*, 1998a). A high concentration of G_{D3} has been detected in developing tissues (Ando, 1983), as well as in human colostrum, and this latter fact might reflect a biological role in the development of organs such as the intestine in the neonate. A major finding was the detection of previously unreported highly polar gangliosides in human milk (Rueda *et al.*, 1995). This type of ganglioside has been detected in several developing tissues, such as the embryonic chicken brain, syncytiotrophoblast, amnion and placenta (Rueda and Gil, 1998). These gangliosides have also been detected in biological fluids such as amniotic fluid (Rueda *et al.*, 1993) which are in contact with developing tissues, and may thus be present due to being shed from these tissues (Ladisch *et al.*, 1983; Li and Ladisch, 1991). The function of these highly complex gangliosides in developing tissues is unknown, but it has been suggested that they act as mediators of specific types of cell-contact interactions during the early stages of mammalian development (Friedman *et al.*, 1983). Thus, the complex gangliosides in human milk may be shed by the lactating mammary gland, and may play an important role in this organ or in developing infant tissues, particularly the small intestine, during early life. Characteristic expression of G_{D1a} during lactation in the murine mammary gland and in the milk-fat globule has also been described (Momoeda *et al.*, 1995).

13.4 Mechanisms of action of gangliosides

Gangliosides are recognized as regulators of cell membrane organization and functions (for a review see Sonnino and Prinetti, 2010). Most of the functions in

which gangliosides are implicated involve cell–cell recognition during normal differentiation or tissue targeting. It is also well known that gangliosides can be ‘kidnapped’ by pathogenic agents and toxins that use them as unintended receptors, and that this is the main mechanism by which gangliosides can prevent infection. Human-milk gangliosides have been shown to be involved in the inhibition of *Escherichia coli* and *Vibrio cholerae* enterotoxins (Otnaess *et al.*, 1983). This inhibitory action was later attributed to the monosialoganglioside G_{M1} , which has been identified as the unintended mammalian cell surface receptor for these enterotoxins (Laegreid and Otnaess, 1987).

Several studies (Teneberg *et al.*, 1993; Yuyama *et al.*, 1993; Idota and Kawakami, 1995) support the notion that cell surface gangliosides function as ‘unintended’ target receptors for bacterial adhesion in specific tissues; dietary gangliosides are putative decoys that interfere with pathogenic binding. Such compounds could modify the intestinal microflora in the neonate and reduce the infectious capacity of pathogenic bacteria. Other findings also describe that sialylated compounds have growth-promoting effects on bifidobacteria and lactobacilli (Idota *et al.*, 1994; Nakano *et al.*, 2001).

Another notable aspect is the ability of gangliosides to modulate the development or behaviour of cells of the immune system. $G_{D1\alpha}$ and G_{D1} have been reported as differentiation markers for Th2 and Th1 lymphocyte subpopulations, respectively (Ebel *et al.*, 1992). Different studies suggest that gangliosides could be involved in the activation of T cells (Yuasa *et al.*, 1990) and in the differentiation of different lymphocyte subpopulations (Ebel *et al.*, 1992; Nakamura *et al.*, 1995; Taga *et al.*, 1995; Nashar *et al.*, 1996). In fact, anti- G_{D3} antibodies deliver a potent co-stimulatory signal for antigen-induced proliferation of $CD4^+$ T lymphocytes (Schlaak *et al.*, 1995); T-cell activation via G_{D3} results in phospholipase C- γ phosphorylation and initiation of a Ca flux (Ortaldo *et al.*, 1996). These data suggest another mechanism of T-cell activation via a single, non-protein surface moiety. As G_{D3} is one of the most important gangliosides in human milk, and has been involved in the mechanism of activation of T lymphocytes, the supplementation of milk formulas with this ganglioside might contribute substantially to the process of proliferation, activation and differentiation of intestinal immune cells in the neonate.

Through sugar-specific interactions with glycan-binding proteins on opposing cells, gangliosides function as receptors in cell–cell recognition, regulating natural killer cell cytotoxicity via Siglec-7, myelin–axon interactions via Siglec-4 (myelin-associated glycoprotein) and inflammation via E-selectin. Gangliosides also interact laterally in their own membranes, regulating the responsiveness of signaling proteins including the insulin, epidermal growth factor and vascular endothelial growth factor receptors. In these ways, gangliosides act as regulatory elements in the immune system, in the nervous system, in metabolic regulation and in cancer progression (López and Schnaar, 2009).

Gangliosides are also major components of membrane raft domains, which have been proposed to serve as platforms to facilitate cellular signaling, viral entry and cell–cell communication. Dietary gangliosides may modulate the structure and

composition of lipid rafts, potentially influencing a wide range of biological processes, including immune function, neuronal signaling, cancer cell growth, entry of pathogens through the gut barrier and insulin resistance in metabolic disorders. Being part of these rafts domains in several tissues, such as brain, small intestine and adipose tissue, gangliosides may be critical for normal development and function, but they also appear to be involved in pathological processes (Yaqoob and Shaikh, 2010). More recent publications describe that normal composition of gangliosides is essential for the maintenance of lipid rafts and that gangliosides are considered to be involved in the maintenance and repair of nervous tissues (Furukawa *et al.*, 2011; Ohmi *et al.*, 2011). Glycolipid-enriched microdomains/rafts architecture is destroyed by ganglioside deficiency with gradual intensity depending on the degree of defects of their compositions. Implication of inflammation caused by deficiency of gangliosides in various neurodegenerative diseases has also been reported (Ohmi *et al.*, 2012).

Dietary gangliosides, together with polyunsaturated fatty acids (PUFAs) may also be important for retinal development, especially during early infancy. In fact, rats fed dietary gangliosides showed increased total retinal ganglioside content during development. The compositional changes in retinal phospholipids in response to both dietary gangliosides and PUFAs may affect light adaptation and signaling which, in turn, could lead to enhanced retinal development in neonates (Park *et al.*, 2005).

Gangliosides are known to play a role in neuronal growth, migration and maturation, neuritogenesis, synaptogenesis and myelination. In addition to their roles in development and structure of the brain, gangliosides also play a functional role in nerve cell communication (McJarrow *et al.*, 2009). Thus, some animal studies have explored the effects of feeding cholesterol and gangliosides (or the sialic acid Neu5Ac, which is a component of all gangliosides) during early development, and the results of these studies suggest that it is plausible that they can influence brain development as well as development of other systems (Carlson, 2009). The work of Park *et al.* (2005) also suggests that gangliosides could contribute to ganglioside sialic acid content in both the intestine and brain during development. Other recent animal studies have shown the potential for cow milk-derived glycomacropeptide as a source of sialic acid that increases sialylation in brain glycoproteins and positively influences an outcome analogous to cognition in animal models (Wang *et al.*, 2007).

On the other hand, a role of gangliosides in the ramification of microglia has also been described. Microglia are immunologically competent cells in the central nervous system and are considered to be a key player in brain inflammation. Brain gangliosides mixture and G_{M1} induced typical ramification of cultured rat primary microglia, although G_{D1a} and G_{T1b} did not (Park *et al.*, 2008). Also, gangliosides have been shown to stimulate the expression and release of B cell activating factor (BAFF), a recently identified member of the tumor necrosis factor (TNF) ligand superfamily that plays a critical role in B cell differentiation, survival and regulation of Ig production (Kim *et al.*, 2009).

Another report describes that TLR2 participates in the initiation of ganglioside-

triggered inflammatory signaling responses. Using flow cytometry analysis and immunofluorescence microscopy, the authors found that gangliosides rapidly enhanced the cell surface expression of TLR2 in microglia, while reducing that of TLR4. The ganglioside-dependent increase of TLR2 expression was also observed at the messenger RNA and protein levels. Gangliosides stimulate the interaction of TLR2 with Myd88, an adaptor for TLRs, and lipid raft formation was closely associated with the ganglioside-induced activation of TLR2 and subsequent inflammatory signaling. These results collectively suggest that TLR2 contributes to the ability of gangliosides to cause inflammatory conditions in the brain (Yoon *et al.*, 2008).

Very recent reports address the hypothesis that changes in lipid rafts induced by alterations in their ganglioside and/or cholesterol content, or the interaction of mutant protein with them, provide the keys to understanding the onset of neurodegeneration that can lead to dementia or Alzheimer's Disease (AD) (Schengrund, 2010; Yaqoob and Shaikh, 2010). Cell membranes may be the location where the neurotoxic cascade of AD is initiated. Perturbation of membranes, binding to lipids and alteration of cellular calcium signaling by amyloid-beta ($A\beta$) peptides have been reported as potential mechanisms involved (Eckert *et al.*, 2010). Because gangliosides are enriched in neuronal membranes and bind to $A\beta$ and APP (amyloid precursor protein), they are probably involved in the conformational changes in these molecules that are a hallmark of the pathogenesis of AD. Other studies have demonstrated that an increase in the level of G_{M1} results in the aggregation of $A\beta$ *in vitro* and that AD brains expressed unusual deposits called GAb complexes. These observations indicate that neuronal gangliosides are involved in the accumulation of circulating $A\beta$ to form complexes that are expressed in the AD brain. Because it has been demonstrated that G_{M1} possesses neurotrophic properties, administration of G_{M1} may exert beneficial effects in AD. Additionally, G_{M1} infusions may be useful for the sequestration of excess $A\beta$ in AD patients to alleviate their toxic effects (Ariga *et al.*, 2008).

A recent report has also described that obesity causes a shift in metabolic flow of gangliosides in adipose tissue. Obesity led to a dramatic increase in G_{M2} , G_{M1} , and G_{D1a} with the G_{M3} content unchanged. These results suggest which gangliosides are involved in the pathophysiology of obesity-related diseases (Tanabe *et al.*, 2009). Recent results also suggest that insulin resistance, associated with obesity and other metabolic disorders, could involve changes in the dynamics and organization of lipid raft domains with G_{M3} being the individual ganglioside mediating dysfunction of insuling signaling (Langeveid and Aerts, 2009; Yamashita *et al.*, 2003). The link between chronic low-grade inflammation in obesity and metabolic syndrome might directly involve gangliosides in a membrane microdomain-related mechanism.

13.5 Effects of gangliosides on immune function

Our group reported some findings related to the effect of dietary gangliosides on intestinal immunity in mice at weaning. Animals fed with gangliosides showed an

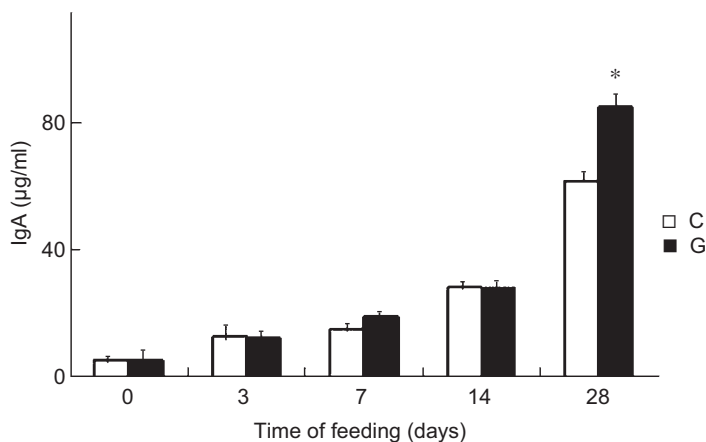


Fig. 13.2 Effect of dietary gangliosides on the luminal content of intestinal IgA in mice at weaning. Concentration of IgA, normalized by intestine length and mouse weight, at the intestinal lumen of mice fed on a control diet (C) or the same diet supplemented with gangliosides, at 47 mg/kg (G). Samples were collected at 3, 7, 14 and 28 days of feeding. Results are means $\pm$ SEM. Two-way ANOVA and Bonferroni test were used to determine the effects of diet and time of feeding as sources of variation (* $P < 0.05$). Source: Rueda R (2007), 'The role of dietary gangliosides on immunity and the prevention of infection', *Br J Nutr*, 98, S68–S73.

earlier development of cytokine-secreting cells, and a higher number of Th1 and Th2 cytokine-secreting lymphocytes in lamina propria and Peyer's patch lymphocytes after four weeks of feeding (Vázquez *et al.*, 2001). Another important finding was that dietary gangliosides increase the number of intestinal IgA-secreting cells (Vázquez *et al.*, 1999) and the luminal content of secretory IgA in weanling mice (Vázquez, *et al.*, 2000), which constitutes the main mechanism of defense against micro-organisms entering through the gastrointestinal tract. Figure 13.2 shows the effect of dietary gangliosides on the luminal content of intestinal IgA in mice at weaning. These results support others published previously reporting that different gangliosides modulate in a positive way the production of immunoglobulins (Kimata, 1994; Kanda and Tamaki, 1998; Kanda, 1999). Ganglioside influences on *in vitro* intestinal lymphocyte proliferation have also been reported (Vázquez *et al.*, 2002).

On the other hand, another publication describes how G_{M3} and G_{D3} differentially inhibit dendritic cell maturation and effector functionalities. Because G_{D3} inhibits dendritic cell functionalities overall more than G_{M3} , it may be concluded that the immune-modulating effect of the ganglioside fraction of breastmilk might be more prominent at the commencement of lactation during which the milk contains the most G_{D3} (Bronnum *et al.*, 2005).

Recent work has described that ganglioside treatment induces a state of tolerance to TLR signaling, leading to blunted activation of innate immune

responses (Shen *et al.*, 2008). The same group has also reported data suggesting that ganglioside-exposed dendritic cells promote regulatory T-cell activity that may have long-lasting effects on the development of tumor-specific immune responses (Jales *et al.*, 2011). Although these results have been reported in the context of tumor studies, they also suggest a role for dietary gangliosides promoting regulatory responses when contacting with dendritic cells at intestinal level.

Our group has also recently studied tolerance-related parameters (incidence and duration of diarrhea, fecal color and consistency) in piglets fed a control or prototype formulas containing fructooligosaccharides and a higher content of sialic acid, gangliosides and phospholipids (Ramirez *et al.*, 2008). Although the incidence of diarrhea was not significantly different among study groups, the duration of diarrhea was shorter in piglets fed with prototype formulas than in piglets fed with control formulas. These results support a role of gangliosides on promoting development of intestinal immunity and, as a consequence, on prevention of infection.

13.6 Effects of gangliosides on inflammation

As mentioned in Section 13.4, Yoon *et al.* (2008) have suggested that gangliosides, through modulation of TLR expression, may contribute to inflammatory conditions in the brain. Nonetheless, a later report has also suggested that ganglioside deficiency may lead to inflammatory reactions and neurodegeneration through complement activation (Ohmi *et al.*, 2009). Consequently, dietary gangliosides might have an anti-inflammatory role also in the brain. On the other hand, gangliosides, through their role in the ramification of microglia, a key player in brain inflammation, also described in Section 13.4, might positively or negatively influence this process, depending on the individual gangliosides considered (Park *et al.*, 2008; Kim *et al.*, 2009).

Other reports also suggest an anti-inflammatory role for gangliosides in the intestine. This bowel-protective effect of gangliosides is indicated by modulation of vasoactive mediators and pro-inflammatory signal suppression. This effect might be particularly relevant for the prevention of necrotizing enterocolitis (NEC), a clinical situation with high morbidity in premature infants (Schnabl *et al.*, 2009a).

This anti-inflammatory role of gangliosides might be related to the ability of dietary gangliosides to modify the composition of the brush border membrane in the intestinal mucosa (Park *et al.*, 2005, 2006; Schnabl *et al.*, 2009b). Ganglioside G_{D3} , the main ganglioside present in colostrum and developing tissues is known to regulate cell growth, differentiation, apoptosis, and inflammation. Feeding a G_{D3} -enriched diet to rats increases G_{D3} in intestinal lipid rafts and blood. The results were dependent on enterocyte G_{D3} uptake time and concentration, with uptake efficiency and fate influenced by route of delivery. G_{D3} uptake across the basolateral membrane was more efficient than apical delivery, and apical G_{D3} was metabolized with some cell retention and transfer, whereas basolateral G_{D3} was mostly metabolized (Schnabl *et al.*, 2009b).

Another study from the same group describes that this increase in ganglioside content decreased the cholesterol and caveolin-1 content of lipid microdomains. Cholesterol depletion in membranes inhibits cellular entry of pathogens and generation of inflammatory signals by disrupting membrane microdomain structure. In addition, the study demonstrated that feeding rats dietary gangliosides results in reduced generation of inflammatory eicosanoids and cytokines by the intestinal mucosa following acute exposure to bacterial endotoxin (Park *et al.*, 2007).

This group has also shown that dietary gangliosides inhibit degradation of gut occludin tight junction protein during lipopolysaccharide (LPS)-induced acute inflammation, suggesting a role of these compounds in protecting the integrity of the intestinal barrier during acute gut inflammation. Dietary gangliosides increased the concentration of the anti-inflammatory cytokine interleukin-10 in response to LPS, thereby inhibiting nitric oxide production and protecting gut occludin tight junction protein from degradation (Park, *et al.*, 2010).

A recent study from this group describes how dietary gangliosides reduce pro-inflammatory signaling in the intestine. Accelerated catabolism of gangliosides in intestinal disease results in increased pro-inflammatory signaling. Restoring proper structure and function to the diseased intestine can resolve inflammation, increase resistance to infection, and improve gut integrity to induce remission of conditions such as NEC and Crohn's disease. Maintaining inactive state of disease may be achieved by reducing the rate at which gangliosides are degraded or by increasing intake of dietary gangliosides (Miklavcic *et al.*, 2012).

13.7 Conclusion and future trends

As far as we know, only one clinical study has been published to date testing the role of dietary gangliosides in humans (Rueda *et al.*, 1998b, Rueda, 2007). In this study developed by our group, we tested the influence of supplementing an adapted milk formula with gangliosides, at a total concentration of 1.43 mg/100 kcal, on the fecal microflora of preterm infants. At all sampling times, feces from infants fed with ganglioside-supplemented formula had significantly lower relative content of *E. coli* than feces from infants fed with control milk formula; the difference was especially significant at age seven days postnatal ($P < 0.001$). At age 30 days postnatal, fecal bifidobacteria counts were higher in infants fed with ganglioside-supplemented formula ($P < 0.05$). This particular impact of gangliosides on *E. coli* and on bifidobacteria is shown in Fig. 13.3. We concluded that gangliosides at concentrations present in human milk significantly modify the fecal flora. However, further studies are required to clarify the mechanisms involved in this action and the relevance of this finding to clinical outcomes in preterm neonates.

Recent studies describe a functional role for gangliosides on the interaction between host and some pathogens, such as *campylobacter jejuni*, HIV or rotavirus (Day *et al.*, 2012; Puryear *et al.*, 2012; Martinez *et al.*, 2013). Whether dietary

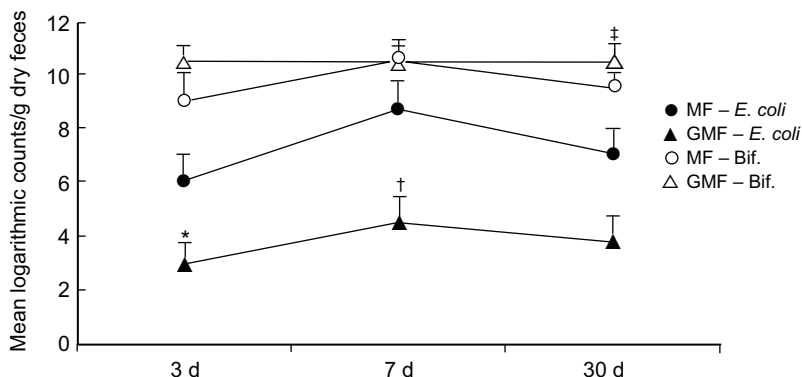


Fig. 13.3 Impact of gangliosides on *E. coli* and on bifidobacteria (Bif). Logarithmic microbial counts in dry feces of preterm newborn infants fed on milk formula (MF) and milk formula supplemented with gangliosides, at 1.43 mg/100 kcal (GMF). Results are means $\pm$ SEM. Twenty samples were analyzed for each feeding group. Samples were collected at 3, 7 and 30 days of life. Kruskal–Wallis and Friedman non-parametric tests were used to determine the effects of diet and postnatal age as sources of variation (* $P < 0.01$ (versus MF group); † $P < 0.001$ (versus MF group); ‡ $P < 0.05$). Source: Rueda R (2007), 'The role of dietary gangliosides on immunity and the prevention of Infection', *Br J Nutr*, 98, S68–S73.

gangliosides might have a role through false receptors interfering with the inner mechanism of action for these pathogens to interact with the host remains to be elucidated.

Apart from this potential role of gangliosides in the prevention of infection, several works developed in animals suggest a role for gangliosides in brain and visual development early in life. The work developed by Park *et al.* (2005), mentioned above in Section 13.4, demonstrated that rats fed dietary gangliosides showed increased total retinal ganglioside content during development. On the other hand, recent reports also support the idea that dietary gangliosides, together with other complex lipids, might be important in supporting brain development early in life. One of these works suggests that maternal supplementation with a complex mixture of lipids, including gangliosides, during pregnancy and lactation had a significant early impact on brain weight and ganglioside and phospholipid content in offspring, although it did not alter long-term behavioral function assessed using standard behavioral techniques (Gustavsson *et al.*, 2010). However, the same authors have also described that supplementation with the same mixture of complex lipids in growing rats resulted in improvements in parameters related to increased learning ability using classic behavioral test platforms (Vickers *et al.*, 2009). Although these works are not conclusive, they suggest that dietary gangliosides, when added to a product for pregnant women or in infant formulas, might support brain and visual development in the offspring. Nonetheless, studies developed in humans are needed to confirm if these effects described in animals also have clinical relevance.

The role of gangliosides in the prevention of inflammation at the intestinal level, described above, suggests that dietary gangliosides might be useful in several

clinical situations in which inflammation is involved in pathogenesis. As mentioned above, this effect might be particularly relevant for the prevention of NEC, a clinical situation with high morbidity in premature infants. The fact that this clinical situation has a lower incidence in breastfed infants than in formula-fed infants, and the beneficial effects demonstrated for dietary gangliosides on the prevention of NEC in animal models simulating this clinical disease, support the idea that gangliosides might be one of the compounds present in human milk that contribute to prevention of inflammation in the neonatal intestine and, consequently, prevent clinical pathologies derived from an increase of pro-inflammatory mediators in the gut (Schnabj *et al.*, 2009a). Nonetheless, a recent work has described that bovine colostrum is superior to ganglioside-enriched formulas in stimulating intestinal function and NEC resistance in a piglet model of preterm infants. The observed results may be due to a synergistic action of various milk bioactives (Møller *et al.*, 2010).

On the other hand, gangliosides, as major components of raft domains, have several roles in many tissues, including the brain, small intestine and adipose tissue, that have been discussed in Section 13.4. As a consequence, alterations in ganglioside composition of lipid rafts can significantly affect cell function. In fact, a number of recent publications suggest that changes in composition of these lipid rafts might have a significant role in the pathogenesis of several neurodegenerative diseases, including dementia or AD (Ariga *et al.*, 2008; Eckert *et al.*, 2010). It is anticipated that as the biological roles of these lipids become more completely defined, the knowledge will be used to develop methods to inhibit the subtle changes in lipid raft composition that over time may lead to neurodegeneration and dementia. Not only would this reduce the medical burden caused by these diseases, it would enhance the quality of life for much in the aging population (Schengrund, 2010).

In summary, although there are a significant number of publications in the scientific literature related to biological effects and mechanism of action of gangliosides in preclinical models, there is a lack of human studies as a proof of concept demonstrating the clinical relevance derived from these compounds when they are incorporated into the diet. Further understanding of the mechanism of action and interaction with other biological compounds will provide clues for designing clinical studies that can support their use in clinical practice in the future.

13.8 Acknowledgments

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13.9 References

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14

Plant foods and inflammatory processes

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Abstract: In addition to nutrients affecting chronic low-grade inflammation, dietary patterns and specific foods significantly modulate inflammatory processes. This chapter focuses on plant foods including whole grain, vegetables and fruits, soy, nuts, coffee and tea. It summarizes current information from human observational as well as human intervention studies and discusses relevant bioactive compounds such as dietary fibre and phytochemicals.

Key words: inflammation, whole grain, dietary fibre, vegetables and fruit, soy beans.

14.1 Introduction

The term ‘plant foods’ includes all foods consumed by humans which originate from plants. This includes cereals (grains), roots, tubers, plantains, vegetables, fruits, pulses (legumes), nuts and seeds, herbs and spices. While there are botanical definitions for the various plant foods, culinary definitions normally determine the use of plant foods in different cultures (IARC, 2003), especially in the case of vegetables and fruits. This chapter gives an overview of the anti-inflammatory potential of the following groups of plant foods:

- whole grains/refined grains
- vegetables and fruits
- soy
- nuts
- coffee and tea.

In recent years, epidemiological studies pointed to a significant association between plant-based diets and a reduced risk for chronic, low-grade inflammation.

In addition, human intervention studies have investigated the anti-inflammatory effects of specific plant foods including whole grain, vegetables and fruits, soy, nuts and seeds, as well as coffee and tea suggesting that inflammatory processes can be modulated by specific plant foods. The active constituents as well as the underlying mechanisms are only partially known. This chapter considers only the literature based on human studies.

14.2 Whole grain and refined grains

The term ‘grain’ applies to cereal and pseudocereal grains including wheat, rye, barley, rice, maize, oat, millet, and buckwheat. The HEALTHGRAIN consortium recently defined ‘whole grain’ as follows:

Whole grains shall consist of the intact, ground, cracked, or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components – the starchy endosperm, germ, and bran – are present in the same relative proportions as they exist in the intact kernel (http://www.healthgrain.org/webfm_send/44).

Most of the published studies in the field of whole grain and inflammation do not give a clear indication of the processing state of the whole grain products. Most whole grains are processed and processing alters nutrient composition and bioactivity; this may explain in part the contradictory findings in the interventional studies with whole grain and wholemeal products. Published studies so far have investigated a narrow and low range of whole grain intake which limits the interpretation of associations between whole grain intake and markers of chronic, low-grade inflammation.

14.2.1 Observational studies

Data from the 1988–1994 US National Health and Nutrition Examination Survey (NHANES III) suggest that a high intake of whole grain is inversely associated with plasma C-reactive protein (CRP) levels (<3.5 servings/day vs >9.7 servings/day) (Ford *et al.*, 2005). The Insulin Resistance Atherosclerosis Study also reported an inverse association between whole grain intake and plasma CRP and plasminogen activator inhibitor-1 (PAI-1) concentrations (Masters *et al.*, 2010). In contrast, Jensen *et al.* (2006) reported no association between a moderate whole grain intake (8.2 g/day vs 43.8 g/day) and the markers of inflammation (CRP, interleukin (IL)-6, and fibrinogen; leptin however, was decreased) in the Health Professionals Follow Up Study. However, moderate bran intake (1.4–13.6 g/day) correlated inversely with CRP, and germ intake (0.3–2.2 g/day) with IL-6 (Jensen *et al.*, 2006). Data from the Nurses’ Health Study showed reduced CRP and soluble tumour necrosis factor (TNF)- α receptor 2 (sTNFr2) concentrations in diabetic women with a moderate intake of whole grain, bran, and cereal fibre (Qi *et al.*, 2006). A higher intake of whole grain (low 0.8 servings/day, high 2.1 servings/

day) within a Mediterranean diet in diabetic women was associated with increased adiponectin concentrations (Mantzoros *et al.*, 2006). Death attributed to inflammatory diseases was reduced by 35% with a high whole grain intake (rarely or never vs ≥ 19 servings/week) in a prospective study with postmenopausal women (Jacobs *et al.*, 2007). Recent data from the Multi-Ethnic Study of Atherosclerosis also suggest that a higher whole grain intake (0.02 servings/day vs 1.39 servings/day) significantly decreases CRP concentrations in elderly subjects (Lutsey *et al.*, 2007). Data from the European Prospective Investigation into Cancer and Nutrition (EPIC) confirm the inverse association between whole grain intake and plasma CRP concentrations (Montonen *et al.*, 2013).

14.2.2 Interventional studies

In one study, obese subjects ($\text{BMI } 35.5 \pm 4.1$ and $36.1 \pm 4.9 \text{ kg/m}^2$) consumed a hypocaloric diet with or without whole grain foods. CRP decreased by 38% (CRP at baseline $5.9\text{--}6.0 \text{ mg/L}$) in the whole grain group compared to the refined grain group, independent of the observed weight loss which occurred in both groups (Katcher *et al.*, 2008). However, plasma concentrations of IL-6 and TNF- α and PAI-1 activity did not change within the 12-week study period. Based on the outcome of this study, the authors concluded that the changes seen in CRP with a whole grain diet were not part of a systemic anti-inflammatory event (Katcher *et al.*, 2008). In a study from Sweden with similar design but only a six-week intervention (whole grain vs refined grain products), whole grain intake was not associated with changes in the concentrations of CRP and IL-6 or in PAI-1 activity in moderately overweight subjects ($\text{BMI } 28 \pm 2 \text{ kg/m}^2$). Baseline CRP concentrations ($2.03\text{--}2.86 \text{ mg/L}$) and BMI were much lower than in the previous study which may explain the different outcomes of these studies (Andersson *et al.*, 2007).

Recent intervention trials with whole grain (60–120 g/day) or wholemeal intake (30–40 g/day) and comparable CRP baseline concentrations also did not observe any changes in plasma markers of inflammation (Brownlee *et al.*, 2010; Tighe *et al.*, 2010). In the study by Brownlee *et al.* (2010), whole grain products did not substitute refined grain products but were consumed in addition to the refined products. Bioprocessing of whole wheat affected the anti-inflammatory potential in a human intervention study when compared to unprocessed whole wheat (Mateo Anson *et al.*, 2011). Bioprocessing modulated the ratio of pro- to anti-inflammatory cytokines during 24 hours after the intake of 300 g whole wheat bread. This study suggests that subtle changes in the processing of whole grains may be relevant for their anti-inflammatory activity, which could in part explain the contradictory findings of the human intervention studies. A recent study investigating the bioactivity of aleurone-rich foods reported significantly reduced plasma CRP concentrations with a daily intake of 27 g aleurone (Price *et al.*, 2012). The aleurone tissue comprises the outermost layer of the endosperm in cereal grains. Because the total dietary fibre intake did not differ between the two groups, this study further highlights the importance of whole grain bioprocessing.

There has been only one study which investigated the acute effects of whole grain products postprandially. Esposito *et al.* (2003) found that replacing a refined-wheat flour pizza by a similar pizza prepared from whole wheat flour resulted in a decreased postprandial concentration of the pro-inflammatory cytokine IL-18 in both non-diabetic and diabetic subjects from Italy (BMI 26.5 ± 1.1 and $26.9 \pm 1.2 \text{ kg/m}^2$).

14.3 Dietary fibre

A major difference between whole grain and refined grain is the content of dietary fibre including all the bioactive compounds associated with it. In the 1999–2000 NHANES in the USA, dietary fibre intake was inversely associated with serum CRP concentrations (odds ratio = 0.49 for 32 g/day vs. 5.1 g/day) in diabetic women ($n = 3920$, Ajani *et al.*, 2004) as well as in subjects with diabetes, hypertension, and obesity ($n = 7891$, King *et al.*, 2005). Similar findings were reported in a cross-sectional study with healthy subjects ($n = 205$) and with subjects with dysmetabolism ($n = 1653$) in Italy (Bo *et al.*, 2006). The inverse association between fibre intake and CRP has been confirmed in another study population after cross-sectional and longitudinal analyses (Ma *et al.*, 2006). In the Seasonal Variation of Blood Cholesterol Levels Study involving 524 subjects, who had multiple measurements of CRP and dietary intake, dietary fibre intake was inversely related to CRP levels in the regression models adjusted for several traditional cardiovascular disease (CVD) risk factors, current infection status, and season of year (Ma *et al.*, 2006). Intake of insoluble fibre, for which whole grain is a major contributor, was mainly associated with a strong reduction of CRP concentrations (odds ratio = 0.32). In contrast, the same authors observed no correlation between dietary fibre intake and blood CRP concentrations with a single measurement at baseline in the Women's Health Initiative Observational Study (Ma *et al.*, 2008). However, IL-6 and TNF- α R2 were significantly reduced in this female population. In a study with diabetic males ($n = 780$) (Health Professionals Follow Up Study), cereal fibre was positively associated with adiponectin levels after controlling for a number of confounding factors (Qi *et al.*, 2005).

In summary, based on a number of epidemiological studies, whole grain intake is inversely associated with markers of inflammation. Because bran and germ were also effective, this emphasizes the anti-inflammatory potential of the whole grain. Preliminary information suggests a dose–response relationship for whole grain intake and biomarkers of inflammation. Ford *et al.* (2005) used a threshold of 3.5 servings/day and reported linearity between CRP concentrations and intake of dietary fibre for up to 10 servings/day. A minimal dose, however, has not been defined yet. Each serving of whole grain is estimated to reduce plasma CRP concentration by 7% (Lefevre and Jonnalagadda, 2012). Processing status of the whole grain products should be more precisely defined in future studies. Potential mechanisms still have to be elucidated, as well as the active constituents including

dietary fibre, minerals, vitamins, and phytochemicals such as lignans and phenolic acids. The observation that not all but only some biomarkers of inflammation responded to the whole grain intervention may suggest that whole grain does not exert a systemic anti-inflammatory effect.

14.4 Vegetables and fruits

An overwhelming number of studies have investigated the health effects of vegetables and fruits in humans. Overall, a high intake of vegetables and fruits is associated with a lower risk for the major diseases in Western societies. This prompted the WHO to recommend a daily intake of at least 400 g/day of vegetables and fruit (WHO, 2002). All major diseases are associated with a chronic, low-grade inflammation. Constituents of vegetables and fruits may interfere with the physiological processes leading to chronic, low-grade inflammation. A number of observational and intervention studies have addressed the anti-inflammatory potential of vegetables and fruits. A common problem of these studies, however, is the different range of intakes of vegetables and fruits investigated and the extremely variable classifications of low and high intakes.

14.4.1 Observational studies

A cross-sectional study revealed that in healthy elderly subjects (BMI 27.2–28.4 kg/m²) a high intake of vegetables and fruits (five servings/day) was associated with significantly lower concentrations of CRP. For each serving of vegetable and fruit intake, the multiple-adjusted odds ratio for high-plasma CRP was 0.79, suggesting a dose–response relationship (Gao *et al.*, 2004). However, plasma CRP concentrations were assessed with a low-sensitivity assay limiting the interpretation of these results. Data from the 1999–2002 US NHANES suggest that a high intake of vegetables (0 g/day vs >280 g/day) and apples (0 g/day vs >138 g/day) is inversely associated with plasma CRP levels (Chu *et al.*, 2008). Another cross-sectional study with moderately overweight women (BMI 24.9–29.9 kg/m²) reported that a high intake of fruits (362 ± 6 g/day) or vegetables (279 ± 7 g/day) reduced plasma CRP concentrations by 34% and 30%, respectively (Esmailzadeh *et al.*, 2006). In addition, in elderly men (BMI 26.7 ± 3.6 kg/m²) fruit intake was inversely associated with plasma concentrations of CRP and of tissue plasminogen-activator (t-PA) antigen. Vegetable intake was significantly inversely correlated only with t-PA antigen (Wannamethee *et al.*, 2006). The inverse association remained significant even after adjustment for plasma vitamin C. Therefore, the authors concluded that other constituents in fruits besides vitamin C may exert protective effects against inflammation.

In a cross-sectional study from Portugal, the intake of vegetables and fruits was negatively associated with CRP levels in men, while no significant associations were found for women (Oliveira *et al.*, 2009). No clear explanation was given for the observed sex-differences. Another study with elderly females and males at

high risk for cardiovascular disease reported a less clear outcome (Salas-Salvado *et al.*, 2008). This study reported associations between typical Mediterranean food groups and specific inflammation markers. While a high fruit intake was inversely associated with plasma IL-6 concentrations, other biomarkers, CRP, soluble vascular cell adhesion molecule-1 (sVCAM-1), and soluble intercellular adhesion molecule-1 (sICAM-1) were not changed. Vegetable intake was not associated with any of the inflammation markers. However, the tertiles with the lowest fruit (<275 g/day) and vegetable (<402 g/day) intakes were already at very high intake levels as compared to all other studies, and this may have masked a protective effect compared to lower intakes (Salas-Salvado *et al.*, 2008).

Adiponectin is an adipose tissue-derived hormone that appears to be anti-inflammatory. Plasma adiponectin concentrations were positively correlated with the intake of fruits in a cross-sectional study in healthy women from Greece (Yannakoulia *et al.*, 2008), further supporting the hypothesis that a high intake reduces chronic low-grade inflammation. Similar effects were seen in adolescents: vegetable and fruit intake was inversely associated with blood levels of CRP, TNF- α , and IL-6 (Holt *et al.*, 2009). Further information on mRNA expression of some pro-inflammatory markers in young adults was published recently. Individuals in the highest tertile of vegetable and fruit intake had statistically lower values for mRNA expression of sICAM-1, IL-1 receptor antagonist, IL-6, TNF- α , and nuclear factor kappa B1 (NF κ B1) (p50) in peripheral blood mononuclear cells (Hermsdorff *et al.*, 2010).

Besides the quantity of vegetables and fruits consumed regularly, the variety of vegetables and fruits may further modulate inflammatory processes. A recent cross-sectional study in obese Puerto Rican adults reported that vegetable and fruit variety (18 vs 34 different vegetable and fruit varieties per month) but not vegetable and fruit quantity was inversely associated with CRP. The adjusted odds for a high plasma CRP concentration in the tertile with the highest number of vegetable and fruit varieties was 0.68 (Bhupathiraju and Tucker, 2011). Unfortunately, the authors did not identify the anti-inflammatory potential of specific varieties. Based on the outcome of this study, future investigations in this field should assess quantity and variety. The significance of the variety points to the relevance of either a large or a specific spectrum of bioactive constituents such as phytochemicals and dietary fibre to induce anti-inflammatory effects.

14.4.2 Interventional studies

Besides the cross-sectional studies, several intervention studies have looked at the anti-inflammatory potential of vegetables and fruits under more controlled conditions. A six-week intervention study in healthy subjects (BMI 22.1–23.1 kg/m²) with vegetables and fruits (810 vs 221 g/10 MJ) had no effect on CRP and sICAM-1 concentrations (Freese *et al.*, 2004). A randomized controlled four week trial in non-smoking healthy men (BMI 23.8–24.3 kg/m²) showed that subjects with a high consumption (eight servings/day) of carotenoid-rich vegetables and fruits had significantly reduced CRP levels compared with those consuming only two

servings/day (Watzl *et al.*, 2005). Plasma CRP correlated inversely with plasma concentrations of β - and α -carotene but not with other carotenoids (Watzl *et al.*, 2005). Vegetables added to a single high-saturated fat meal partially restored the endothelium-dependent vascular responses observed after consuming such a meal without vegetables (Esposito *et al.*, 2003).

A number of studies investigated more specifically the effect of defined vegetable and fruit varieties. In a randomized, placebo-controlled trial in overweight subjects (BMI 29.1 ± 3.1 kg/m²) supplementation with garlic powder (2.1 g/day) equivalent to 5.2 g/day of fresh garlic for up to 12 weeks did not affect a range of inflammatory biomarkers (CRP, TNF- α and endothelial markers of inflammation) (van Doorn *et al.*, 2006). Supplementation with lyophilized grape powder for four weeks decreased plasma TNF- α in obese pre- and postmenopausal women, while IL-6 and CRP were not changed (Zern *et al.*, 2005). Another small study looked at the effect of concentrated red grape juice on inflammation. In this single-blind, uncontrolled study with haemodialysis patients only the concentration of monocyte chemo-attractant protein (MCP)-1 was decreased, while CRP and adhesion markers were not affected (Castilla *et al.*, 2006). The consumption of sweet cherries (280 g/day, providing 378 mg total phenolics) for four weeks significantly decreased plasma concentrations of CRP (-25%) and RANTES (-21%) in healthy adults (BMI 26.3 ± 0.9 kg/m²) (Kelley *et al.*, 2006). Plasma concentrations of IL-6, sICAM-1 and tissue inhibitor of metalloproteinases-2 did not change during this study. The combined daily intake of fruit juices (250 ml orange and 250 ml blackcurrant juices) for four weeks also reduced CRP and fibrinogen in plasma (both changes were highly correlated), suggesting a common underlying anti-inflammatory process. However, other inflammation markers were not affected (IL-6, PAI-1) (Dalgard *et al.*, 2009). The intake of a frozen sea buckthorne puree by healthy volunteers for 90 days significantly decreased plasma CRP concentrations (Larmo *et al.*, 2008). No other markers of inflammation were investigated in this study. In contrast, orange juice alone (500 ml/day) did not affect inflammation markers in healthy overweight volunteers (Morand *et al.*, 2011).

Bilberry consumption (330 ml/day, four weeks) decreased plasma concentrations of CRP, IL-6, and IL-15, but TNF- α was slightly increased compared to the intake of water in subjects with increased risk for CVD (Karlsen *et al.*, 2010). The antioxidant status (FRAP, TRAP, ORAC) of the study subjects did not differ between groups. In a previous study in healthy adults (BMI 24-25 kg/m²), these authors investigated the effect of supplementation with a bilberry extract providing 300 mg anthocyanins/day (equal to 100 g of fresh bilberries). The extract reduced plasma concentrations of several NF κ B-related pro-inflammatory cytokines (IL-8, RANTES, and interferon- α). In addition, NF κ B-inducing cytokines (IL-4, IL-13) tended to differ from controls, while plasma CRP concentrations did not change (Karlsen *et al.*, 2007). Blueberry consumption (50 g/day freeze-dried equivalent to 350 g fresh blueberries) for eight weeks in subjects with the metabolic syndrome had no effect on a range of inflammation markers (Basu *et al.*, 2010). A similar outcome was observed in healthy subjects supplemented with an elderberry extract (500 mg anthocyanins/day) for 12 weeks (Curtis *et al.*, 2009).

Two studies from Spain investigated the impact of high-pressured orange juice and of a vegetable soup on biomarkers of inflammation. In these uncontrolled studies, orange juice did not modulate CRP concentrations in blood, while the vegetable soup decreased MCP-1 with no further effects on other biomarkers (Sanchez-Moreno *et al.*, 2003, 2004).

14.4.3 Phytochemicals in vegetables and fruits

Phytochemicals which occur primarily in vegetables and fruits, such as the carotenoids, may contribute to the anti-inflammatory potential of these foods. Several studies have focused on the anti-inflammatory potential of carotenoids. In a cross-sectional study in healthy adults (mean BMI 24.1 kg/m²), high intakes of carotenoids were inversely related to plasma sICAM-1 (lutein, lycopene) and CRP (lutein, lycopene, β -carotene) concentrations (van Herpen-Broekmans *et al.*, 2004). Plasma concentrations of β -carotene have also been inversely associated with CRP and IL-6 concentrations and positively correlated with adiponectin concentrations in a cross-sectional study from Japan (Suzuki *et al.*, 2010). Recent data from the Women's Health Study reported that high plasma CRP concentrations were associated with significantly reduced plasma concentrations of α - and β -carotene (Wang *et al.*, 2008). Similar results were reported from NHANES III for carotenoids and CRP (Kritchevsky *et al.*, 2000; Erlinger *et al.*, 2001). However, the use of latex-enhanced nephelometry instead of a high-sensitivity ELISA, and the fact that 74% of the population had levels below the assay's detection limit, limits the interpretation of their results.

Overall, results from cross-sectional studies are in line with data from recent prospective studies. In one prospective study with young adults (BMI 23–25 kg/m²) a high intake of carotenoids (α -carotene, β -carotene, zeaxanthin/lutein, β -cryptoxanthin) was beneficially related with plasma CRP and sICAM-1 concentrations after seven and 15 years of follow-up (Hozawa *et al.*, 2007). In a second prospective study a high intake of carotenoids in elderly (BMI 28.8 $\pm$ 6.8 kg/m²) was associated with lower plasma levels of the pro-inflammatory cytokine IL-6, while α -tocopherol did not correlate significantly (Walston *et al.*, 2006). Two prospective studies reported a lower risk of inflammatory polyarthritis in subjects with a high intake of β -cryptoxanthin (Cerhan *et al.*, 2003, Pattison *et al.*, 2005). In patients with coronary artery disease plasma concentrations of β -carotene, but not lycopene, were also inversely correlated with plasma CRP (Jonasson *et al.*, 2003).

In an intervention study with carotenoid-rich vegetables and fruits, plasma concentrations of α - and β -carotene but not other carotenoids were inversely associated with plasma CRP concentrations in healthy men (BMI 23.8–24.3 kg/m²) (Watzl *et al.*, 2005). However, supplementation of 30 mg/day lycopene (Lyc-O-Mato®) for four weeks had no effect on CRP, IL-6, and TNF- α plasma concentrations (Markovits *et al.*, 2009). A strong decrease in plasma CRP concentration was observed after eight weeks of 15 mg/day lycopene (Lyc-O-Mato®) in healthy subjects with an already low baseline CRP value (Kim *et al.*, 2011). This

suggests that carotenoids may exert specific anti-inflammatory effects. Besides carotenoids, the flavonoids in vegetables and fruits are also potent anti-inflammatory constituents. The different chemical classes of flavonoids and their bioactivities are discussed in Chapter 15.

14.5 Soy

Several randomized controlled interventional trials have shown that supplementation with different quantities of soy protein (20–40 g/day) did not affect markers of inflammation (CRP, IL-6, IL-18, sICAM-1, sVCAM-1, sE-selectin) (Blum *et al.*, 2003; Teede *et al.*, 2004; Hilpert *et al.*, 2005; Campbell *et al.*, 2006; Hanson *et al.*, 2006; McVeigh *et al.*, 2006; Ryan-Borchers *et al.*, 2006; Azadbakht *et al.*, 2007; Matthan *et al.*, 2007; Greany *et al.*, 2007; Charles *et al.*, 2009). Variations in soy protein isoflavone contents did not modulate the outcome in these studies. In agreement, one study reported reduced plasma CRP, TNF- α , and IL-18 concentrations in postmenopausal Iranian women with metabolic syndrome consuming soy nuts but not soy protein in a study lasting eight weeks (Azadbakht *et al.*, 2007).

The major difference between soy protein and soy nuts (comparable contents of isoflavones) was a much higher content of polyunsaturated fatty acids (PUFA) in the soy nuts, suggesting that, rather than soy-specific constituents, the increased intake of PUFA may be responsible for the reduction in biomarkers of inflammation (Azadbakht *et al.*, 2007). The same group investigated in diabetic patients with nephropathy the anti-inflammatory effect of textured soy protein compared with animal protein after four years of intake. Consumption of soy protein significantly reduced plasma concentrations of CRP (Azadbakht *et al.*, 2008). In another study investigating soy nuts in normotensive postmenopausal women, no effect on CRP, IL-6, sICAM-1, and matrix metalloproteinase-9 was observed compared to the control diet (Nasca *et al.*, 2008). However, in hypertensive women levels of sVCAM-1 were significantly lower after soy nut supplementation (Nasca *et al.*, 2008). Again, the increased PUFA intake may have caused this effect.

Long-term soy exposure of premenopausal women was also not associated with anti-inflammatory effects measuring the serum or plasma inflammation markers CRP, IL-6, leptin, and adiponectin (Maskarinec *et al.*, 2009a). Short-term soy intervention in postmenopausal women with or without exercise-induced inflammation had no effect on inflammation markers (IL-1 β , IL-6, TNF- α) (Beavers *et al.*, 2009, 2010). Among men, soy intervention also had no effect on inflammatory status (leptin, MCP-1, MIP-1 β , resistin, adiponectin, TNF- α , IL-6, IL-15, CRP) (Maskarinec *et al.*, 2009b; Zemel *et al.*, 2010; Napora *et al.*, 2011).

Overall these data suggest that (i) soy protein does not affect markers of inflammation, (ii) soybean processing may affect the anti-inflammatory potential of soybean constituents, and (iii) the health status of the study subjects might determine the anti-inflammatory efficacy of specific foods. Replacing animal proteins with plant proteins is beneficial in reducing inflammation indicating an indirect effect of soy protein.

14.6 Nuts

Nuts have been part of several dietary intervention studies (Mediterranean diets, Portfolio diets) which mostly reported a reduced inflammatory status in subjects consuming such diets (Kris-Etherton *et al.*, 2008). However, such studies do not allow any conclusions to be drawn about specific effects of nuts. So far, only a few studies have investigated the effect of specific nuts. The Multi-Ethnic Study of Atherosclerosis reported that a high intake of nuts and seeds (≥ 5 times/week) compared to a low intake (rarely or no consumption) was inversely associated with plasma concentrations of CRP, IL-6 and fibrinogen (Jiang *et al.*, 2006). In contrast, data from the Nurses' Health Study suggest that nut intake is not associated with inflammation markers including sTNFr2, CRP, fibrinogen, sICAM-1, and sE-selectin (Li *et al.*, 2009).

An eight-week intervention with walnuts or cashew (63–108 g/day) in subjects with metabolic syndrome also observed no effect on serum CRP concentrations (Mukkudem-Petersen *et al.*, 2007). A pistachio intervention (60–100 g/day providing about 20% of total energy) for four weeks in healthy young students had no effect on plasma CRP and TNF- α concentrations, while the concentration of IL-6 was significantly reduced (Sari *et al.*, 2010). A recent intervention study with two doses of almonds (10 and 20% of total energy) in healthy adults reported reduced serum concentrations of sE-selectin and CRP, but IL-6, fibrinogen, and tissue plasminogen antigen were not affected (Rajaram *et al.*, 2010). No clear dose-response relationships were observed. In contrast, an earlier study in hyperlipidaemic subjects, also providing two doses of almonds, did not observe an effect on CRP (Jenkins *et al.*, 2002). A systematic review on walnut consumption and inflammation markers also reported inconsistent results for plasma CRP, while walnuts added to a Mediterranean diet resulted in significantly lower sVCAM-1 concentrations (Banel and Hu, 2009). This suggests that, rather than a general anti-inflammatory effect, walnuts may exert anti-inflammatory effects primarily in the endothelium. This is in line with the reduced risk of CVD associated with frequent nut consumption which has been reported in several studies (Kris-Etherton *et al.*, 2008). Major contributors to the anti-inflammatory activity of nuts are polyunsaturated fatty acids, magnesium, and phytochemicals including ellagic acid (Ros, 2009).

14.7 Coffee and tea

Coffee consumption is associated with a reduced risk for various inflammatory diseases including CVD (Andersen *et al.*, 2006). Whether coffee constituents exert these beneficial effects through anti-inflammatory processes has been investigated in only a few studies. One cross-sectional study reported that coffee consumption (>200 ml/day) significantly increased a range of biomarkers of inflammation (Zampelas *et al.*, 2004). However, other cross-sectional studies reported either no effect of coffee consumption on inflammation status in healthy subjects (de Bacquer *et al.*, 2006; Lopez-Garcia *et al.*, 2006) or a reduction of inflammation biomarkers in healthy or diabetic subjects (Lopez-Garcia *et al.*, 2006; Kotani *et al.*,

2008; Williams *et al.*, 2008). One case-control study also observed an inverse association between coffee consumption and a range of inflammation biomarkers (Schulze *et al.*, 2005). A recent intervention study providing two doses of coffee (four and eight cups of filtered coffee/day) confirmed in part the observations from the cross-sectional studies. While IL-18 (decreased) and adiponectin (increased) were significantly modulated by coffee consumption, CRP, leptin, serum amyloid A (SAA), IL-6, macrophage inhibiting factor (MIF), and IL-1ra were not affected (Kempf *et al.*, 2010). No dose-response relation was seen in this study. Plasma caffeine concentrations were positively associated with plasma adiponectin concentrations (Kempf *et al.*, 2010).

Overall, data on the effect of coffee on inflammation are inconsistent. Since coffee can be produced and consumed in so many different ways with large variations in the content of bioactive constituents, future studies should provide more detailed data on the type of coffee consumed and on the bioavailability of the major coffee constituents.

The effect of tea consumption (green and black tea) has been investigated in several small intervention studies ($n = 12-66$), usually with a duration of four weeks. All studies clearly indicate that tea consumption has no significant effect on the inflammatory status (de Maat *et al.*, 2000; Hodgson *et al.*, 2001; Sung *et al.*, 2005; Widlansky *et al.*, 2005; Ryu *et al.*, 2006; Mukamal *et al.*, 2007; Fukino *et al.*, 2008). However, a longer supplementation period of six weeks with black tea in healthy non-smoking men reduced plasma CRP concentrations significantly (Stephoe *et al.*, 2007). Whether the longer intervention period caused this effect has to be investigated in further studies. A cross-sectional study from Japan reported no effect of green tea consumption on inflammation markers (Maki *et al.*, 2010), while a cross-sectional study from Belgium detected significantly lower CRP and SAA concentrations in regular tea drinkers after multivariate analysis (de Bacquer *et al.*, 2006). Overall, there are no consistent data indicating that tea consumption (black, green) has a beneficial effect on the inflammatory status in humans.

14.8 Mechanisms contributing to the anti-inflammatory effects of plant foods

There is no single mechanism which mediates the anti-inflammatory effects of plant foods. Plant foods contain many different bioactive compounds which all target specific steps or molecules in the process of chronic, low-grade inflammation. These bioactive compounds include dietary fibre, PUFA, mineral and trace elements, vitamins, and phytochemicals. Several chapters in this book focus on these bioactive compounds and they will not be discussed in further detail in this chapter. However, dietary fibre, a major constituent of all plant foods, and phytochemicals will be briefly discussed.

For phytochemicals, knowledge concerning the mechanisms of action at the cellular level is limited (Watzl and Leitzmann, 2012). There are many *in vitro* studies using chemical forms and dosages of phytochemicals which do not occur

in plant foods or in human tissues. Most studies were carried out with single phytochemicals (often as aglycones) at rather high concentrations. Phytochemical classes with anti-inflammatory activity include flavonoids, carotenoids, monoterpenes, and sulphides (Watzl, 2008). It is currently unknown how the simultaneous intake of multiple phytochemicals, as occurs with the intake of plant foods, affects immunoregulatory processes.

For flavonoids, the largest group of phytochemicals subject to intensive research activities in recent years, several regulatory processes affecting cell signalling (e.g. activation of NF κ B) have been described (Rahman *et al.*, 2006). Besides modulation of signal transcription, phytochemicals have direct effects on enzyme activities in immune cells resulting in reduced production of inflammatory lipid mediators (prostaglandins, leukotrienes) and cytokines (IL-6, TNF- α). Phytochemicals may also stimulate the synthesis of anti-inflammatory mediators (Garcia-Lafuente *et al.*, 2009; Pan *et al.*, 2009). Phytochemicals further contribute to the control of oxidative stress, which is associated with hyperglycaemia and other diseases characterized by chronic, low-grade inflammation. Again, NF κ B, as a redox-sensitive transcription factor, is a key target for phytochemicals with antioxidative potential such as the flavonoids and carotenoids.

Dietary fibre is a major constituent of all plant foods. Several studies suggest that dietary fibre may affect pro- and anti-inflammatory markers through different mechanisms. Dietary fibre is fermented in the large intestine resulting in the production of short-chain fatty acids (SCFA; acetate, propionate, butyrate), potential key factors in dietary fibre's anti-inflammatory activity. SCFA are rapidly absorbed in the large intestine and some are transported to the liver. Their anti-inflammatory properties have been studied *in vitro* and in animal models, but human studies are limited. The SCFA butyrate inhibited *in vitro* mRNA expression of pro-inflammatory cytokines by interfering with the signal transcription factor NF κ B (Segain *et al.*, 2000). All three SCFA decreased lipopolysaccharide (LPS)-induced TNF- α release from neutrophils (Tedelind *et al.*, 2007). Recent studies suggest that SCFA are sensed through G protein-coupled receptors on immune cells (GPR41 and 43). The importance of these receptors for the control of inflammation processes has been demonstrated in several animal studies (Maslowski *et al.*, 2009). GPR43-knockout mice showed exacerbated inflammation in different models of inflammatory diseases compared to wild-type mice. Stimulation of GPCR43 by SCFA was necessary for the normal resolution of inflammatory responses (Maslowski *et al.*, 2009). However, it is presently not clear whether the intake of dietary fibre with plant foods results in physiologically relevant concentrations of SCFA in the periphery. Blood acetate concentrations at least are well within the active range to stimulate immune cells via GPCR43 (Nilsson *et al.*, 2003). In addition, SCFA seem to have the potential to prevent infiltration of immune cells from bloodstream into the adipose tissue (Meijer *et al.*, 2010), a major site for the production of IL-6. Both SCFA receptors, GPR41 and GPR43, are expressed by human adipose tissue (Al-Lahham *et al.*, 2010).

Dietary fibre may also inhibit inflammation through its effects on lowering blood glucose levels. Hyperglycaemia is known to increase concentrations of pro-

inflammatory cytokines (North *et al.*, 2009) and to induce oxidative stress which directly affects redox-sensitive transcription factors. Colonic metabolites of dietary fibre-associated phytochemicals, such as ferulic acid, exert anti-inflammatory effects *in vitro* and may further contribute to the *in vivo* activity (Mateo Anson *et al.*, 2011).

Besides open questions related to the effects of plant food constituents, the information about the process of chronic, low-grade inflammation is also not completely clear. A frequent observation is that plasma concentrations of CRP have changed during a dietary intervention, although the key cytokine for the induction of hepatic CRP production, IL-6, had not changed. This is in line with earlier reports that IL-6 is not the only determinant of plasma CRP (Weinhold and Rüther, 1997). Which regulators besides IL-6 control CRP and are responsive to dietary interventions is currently not known. In general, pro-inflammatory cytokines in plasma may be derived from a number of different tissues and cells with variable time courses in response to a dietary intervention. Often, only one plasma sample from one time-point has been collected, preventing a clear picture of the overall changes in the production of inflammatory markers. This could further explain why not all pro-inflammatory plasma markers respond in a similar way.

14.9 Conclusion

This review suggests that plant foods have an anti-inflammatory potential. The effect, however, is not a general phenomenon but primarily relates to specific plants including whole grain and vegetables and fruits. Before this knowledge can be used for humans to support the prevention of chronic, low-grade inflammation, more precise information is needed about the role of plant food varieties, of processing, and of quantities which are effective in humans. Future studies should include a broader spectrum of biomarkers and the optimal set of biomarkers still has to be defined.

14.10 References

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15

Flavonoids and immune function

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Abstract: It has been suggested that flavonoids, secondary metabolites widely present in the vegetable kingdom, are involved in the role played by plant foods in disease prevention. The immune-modulating action of flavonoids *in vitro* suggests a specific role in modulating cytokines and transcription factors such as nuclear factor kappa B (NFκB). However, the majority of studies have been conducted with non-physiological concentrations of flavonoids and without considering the nature of the metabolites found in the bloodstream. In humans, the number of studies is limited and the results are contrasting, leaving open the debate about the role of flavonoids as modulators of immune response.

Key words: flavonoids, bioavailability, immune function, human, plant foods, inflammation.

15.1 Introduction

A diet rich in plant foods has been shown to be protective against the development of inflammatory diseases (Calder *et al.*, 2009; Trichopoulou *et al.*, 2009). In recent years, attention has been focused on increasing understanding of the biological role of molecules, such as flavonoids, contained in high amounts in plant foods such as soy, wine, cocoa, fruits and vegetables. This research has suggested a role for these molecules in the modulation of the immune response. The actions described *in vitro* involve the inhibition of production of certain cytokines and adhesion molecules amongst other mediators of immune and inflammatory interactions (Santangelo *et al.*, 2007). Molecular mechanisms include inhibition of transcription factors such as nuclear factor kappa B (NFκB) and activating

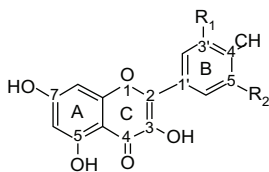
protein-1 (AP-1), as well as activation of nuclear factor erythroid 2-related factor 2 (Nrf2) (Gopalakrishnan and Kong, 2008). However, this *in vitro* evidence is not always supported by *in vivo* results: the low extent of absorption of flavonoids into the circulatory stream and the extensive metabolic activity during absorption lead to molecules with different chemical structure and activity compared to the compounds originally present in the foods (Manach and Donovan, 2004). Thus, the majority of *in vitro* evidence has been obtained for flavonoid compounds, which are present in plant foods, disregarding the biological metabolites found *in vivo*. Moreover, the concentrations to which cells have been exposed have often been far higher than those measured in biological fluids, raising doubts about the relevance to *in vivo* situations. In this chapter we will review the evidence regarding the bioavailability and metabolism of flavonoids and their role as dietary modulators of the immune system.

15.2 Structure, classification and sources of flavonoids

Flavonoids are a wide group of almost 5000 secondary plant metabolites, sharing a common carbon skeleton of two benzene rings (ring A and B), joined by a 3-carbon bridge (C6–C3–C6) (Fig. 15.1). They occasionally occur in plants as aglycones, but the majority exists naturally as glycosides. Hydroxyl groups are usually present at the 4', 5- and 7-positions of the carbon skeleton, while methyl and isopentyl units are less frequent substituents. The flavonoids group comprises diverse subclasses, among which flavonols, flavones, flavanols, anthocyanidins, flavanones and isoflavones are the most important dietary compounds (Fig. 15.1). The main food sources are fruits, vegetables, grains, tea, coffee and wine. The distribution in foods and structural differences of flavonoids are well documented and the latest advances in these aspects have been reviewed by several authors in the past decade (Aherne and O'Brien, 2002; Manach and Donovan, 2004; D'Archivio *et al.*, 2007; Crozier *et al.*, 2009; Passamonti *et al.*, 2009; Pérez-Jiménez *et al.* 2010). Table 15.1 shows the flavonoid content of fruits, vegetables, cocoa, wine, teas and soy. Ranges of concentration in the table and values described in the text refer to the latest published database, Phenol-Explorer (<http://www.phenolexplorer.en>) (Neveu *et al.*, 2010; Pérez-Jiménez *et al.*, 2010).

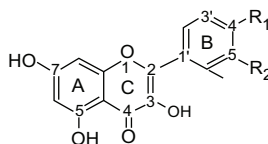
15.2.1 Flavonols

Flavonols are the most widespread flavonoids in food. The main dietary flavonols are quercetin and kaempferol, naturally found associated to sugar moieties, mostly glucose or rhamnose. Good dietary sources are spinach and onions, with concentrations as high as 120 and 160 mg/100 g, respectively. Other good sources include different kinds of berries, broccoli and dark chocolate (20–40 mg/100 g), tea and red wine (5–20 mg/100 ml) (Table 15.1).



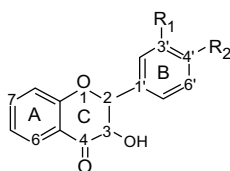
Flavanols:

Kaempferol	$R_1 = H, R_2 = H$
Quercetin	$R_1 = OH, R_2 = H$
Isorhamnetin	$R_1 = OCH_3, R_2 = H$
Myricetin	$R_1 = OH, R_2 = OH$



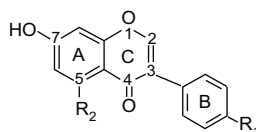
Flavones:

Apigenin	$R_1 = OH, R_2 = H$
Luteolin	$R_1 = OH, R_2 = OH$



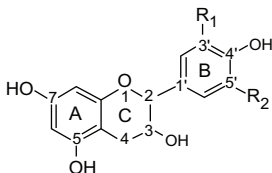
Flavanones:

Naringenin	$R_1 = H, R_2 = OH$
Hesperetin	$R_1 = OH, R_2 = OCH_3$



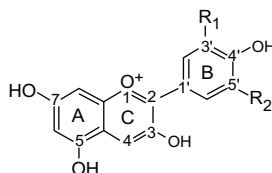
Isoflavones:

Genistein	$R_1 = OH, R_2 = OH$
Daidzein	$R_1 = OH, R_2 = H$



Flavanols:

(+)-Catechin	$R_1 = OH, R_2 = H$
(+)-Gallocatechin	$R_1 = OH, R_2 = OH$
(-)-Epicatechin	$R_1 = OH, R_2 = H$
(-)-Epigallocatechin	$R_1 = OH, R_2 = OH$



Anthocyanins:

Cyanidin	$R_1 = OH, R_2 = H$

Fig. 15.1 Chemical structures of different flavonoid subclasses. Modified from Passamonti (2009) and Manach (2004).

15.2.2 Flavones

Flavones are mainly represented by luteolin and apigenin glycosides. Good dietary sources are artichokes and black olives (up to 60 mg/100 g), celery leaves (around 130 mg/100 g) and whole grain wheat flour (around 80 mg/100 g). They are also found in citrus skin, as polymethoxylated hydrophobic forms.

15.2.3 Flavanones

The best-known flavanone compounds are the aglycone naringenin, hesperetin and eriodictyol. They are generally glycosylated in the C7 position, by a

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Table 15.1 Ranges of flavonoid subclass concentrations in plant foods and their derivatives

	Total concentration of flavonoids sub-classes (mg/100 g or mg/100 ml)						
	1–5	5–20	20–40	40–60	60–80	80–100	>100
Flavonols	Almond	Black bean	Asparagus			Black chokeberry	Red onion
	Apple juice	Blackcurrant	Bluberry			Yellow onion	Spinach
	Apricot	Black tea	Broccoli				
	Bilberry	Blackberry	Cranberry				
	Black grape	Green tea	Dark chocolate				
	Green bean	Pecan nut	Endive				
	Green grape	Pistachio	Lingonberry				
	Hazelnut	Plum	Wholegrain				
	Lettuce	Red lettuce	buckwheat flour				
	Strawberry	Red wine					
	White onion						
	Zucchini						
Flavones	Extra virgin olive oil	Blond orange juice		Black olive		Wholegrain wheat flour	Celery
	Lemon juice	Verbena tea		Globe artichoke			
	Red lettuce						
Flavanones		Lime juice	Lemon juice	Blond orange juice			
		Pummelo juice		Grapefruit juice			

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Isoflavones

Soy sauce	Soy milk Soybean sprouts	Tofu		Soy meat	Soy yogurt	Soy flour Soy nuts Tempe

Flavanols

Cashew nut	Avocado	Apple juice	Peach	Black grape	Green grape	Almond
Cloudberry	Banana	Apricot	Redcurrant	Black tea		Apple
Green bean	Kiwi	Black elderberry	Red wine	Green tea		Blackcurrant
Pear juice	Mango	Blackberry		Walnut		Blueberry
Persimmon	Nectarine					Cocoa powder
Rosé wine	Red raspberry					Dark chocolate
White wine	Sherry					Hazelnut
	Sweet cherry					Pecan nut
						Pistachio
						Plum
						Strawberry

Anthocyanins

Cloudberry	Pomegranate juice	Black bean	Cranberry	Black grape	Black olive	Black chokeberry
Gooseberry		Redcurrant	Lingoberry	Red raspberry		Blackcurrant
Orange juice		Red wine	Plum	Strawberry		Black elderberry
Red lettuce						Blackberry
Red onion						Blueberry

Source: modified from Pérez-Jiménez *et al.* (2010).

disaccharide, mostly neohesperidose, which imparts a bitter taste, or rutinose, which is flavorless. They are present only in small concentrations in the pulp of citrus fruits and in higher concentrations in the white spongy part. Good dietary sources are lemon, orange and grapefruit juices, with concentrations in the range of 5–60 mg/100 ml (Table 15.1).

15.2.4 Isoflavones

Isoflavones have the B-ring attached at the C3 rather than the C2 position, with a hydroxyl group in the C4' position and one in the C7. These structural characteristics confer hormonal properties on them, including the ability to bind to estrogen receptors; hence these compounds are often referred to as phytoestrogens. The best known molecules are genistein and daidzein, which are found almost exclusively in the leguminous plants, in particular in soy and soy products, either as aglycones or more often as glycosides forms. Soy flours (more than 400 mg/100 g), soy nuts (more than 200 mg/100 g) and tempeh (more than 100 mg/100 g) are the richest sources (Table 15.1).

15.2.5 Flavanols

Flavanols, in contrast to other flavonoids, are not glycosylated in foods. They exist as both monomer (catechins) and polymer (proanthocyanidins) forms. Catechins exist in four isomer forms, among which (+) catechin and (–) epicatechin are widely distributed in nature. They can be found hydroxylated, to form gallo-catechins, or undergo esterification with gallic acid. Proanthocyanidins, also known as condensed tannins, are dimers, oligomers and polymers of catechins, which are responsible for the astringent character of fruits. Proanthocyanidins have a wide range of structures and can occur as polymers of up to 50 units. Polymers that consist exclusively of epicatechin units are called procyanidins, and are the most abundant type of proanthocyanidins in plants. So far, the highest concentrations of catechins are found in cocoa (up to more than 3400 mg/100 g), dark chocolate (up to more than 1500 mg/100 g) and berries (between 100 and 600 mg/100 g). Both green and black teas, as well as apples, nuts, grape, peach, apricots and red wine, are also good sources (Table 15.1).

15.2.6 Anthocyanins

Anthocyanins are red, blue or purple pigments found in fruits and flowers. They are found in glycosylated forms of their monomers, which are called anthocyanidins, most of the time with glucose in the C3 position. Anthocyanins are particularly abundant in red fruits, such as dark-colored berries (up to 1300 mg/100 g), and are found in good quantities also in red wine, colored beans, red lettuce and red onions (Table 15.1).

15.3 Bioavailability and metabolism of flavonoids

Extensive reviews of flavonoid metabolism were recently provided by Spencer (2003), Manach and Donovan (2004), Manach *et al.* (2005), D'Archivio *et al.* (2007), Crozier *et al.* (2009), Passamonti *et al.* (2009) and Del Rio *et al.* (2010). Flavonoids, and polyphenols in general, once absorbed, are targeted as xenobiotics and metabolized so as to be eliminated, resulting in a scarce bioavailability and low (nano/micromolar) circulating concentrations post-ingestion. Their metabolism generally starts in the lumen of the small intestine. However, evidence obtained in animal models suggests that absorption at the gastric level for the aglycone forms is also possible (Crespy *et al.*, 2002; Talavera *et al.*, 2003). Metabolic activities have been observed also in the oral cavity, such as degalloylation of flavanol gallate esters prior to absorption through the oral mucosa and specific interactions with proteins before passing into the gastric lumen (Spencer, 2003). As flavonoids, with the exception only of flavanols, are usually found in foods in the form of glycoside conjugates, hydrolysis normally occurs prior to intestinal absorption, by either lactase phloridizin hydrolase (LPH) in the brush border of the small intestine epithelial cells, or by the cytosolic β -glucosidase (CBG) within the epithelial cells. Studies conducted *in vitro* with cellular models have shown that flavonoids undergo an extensive phase II metabolism during transfer from the intestinal lumen to the circulatory stream, resulting in sulfate, glucuronide and/or methylated conjugates (Spencer *et al.*, 1999; Vaidyanathan Walle, 2001).

The degree of metabolic activity is strongly dependent upon the flavonoid structures, such as catechins, that may, to a small degree, also be absorbed intact (Spencer, 2003). Phase II metabolism enzymes, comprising sulfo-transferases (SULT), uridine-50-diphosphate glucuronosyl-transferases (UGTs) and catechol-O-methyl-transferases (COMT), are present in the small intestine as well as at hepatic and renal levels, indicating that conjugation may occur in all of these sites. It has been observed that, while sulfation mainly occurs in the liver, glucuronides and methylated conjugates are primarily generated in the enterocytes, prior to further conjugation in the hepatocytes (Manach *et al.*, 2003).

Not all the flavonoids and related compounds are absorbed in the small intestine. Some of them reach the large intestine, where they undergo metabolism by the colonic microflora (Jaganath *et al.*, 2006). Bacterial enzymes may catalyze many reactions, including hydrolysis, dehydroxylation, demethylation, ring cleavage, decarboxylation and deconjugation, as well as the breakdown of the flavonoid backbone itself to form simple phenolic acids and hydroxycinnamates, which can be absorbed into the circulatory system (Spencer, 2003). Once in the bloodstream, flavonoid metabolites circulate bound to proteins, in particular to albumin, affecting their rate of clearance and their potential delivery to cells and tissues (Manach and Donovan, 2004).

To date, only few data are available regarding polyphenol uptake and concentrations in body tissues, and the mechanisms involved in the cellular influx and efflux of flavonoids are still largely unknown (D'Archivio *et al.*, 2007). Most nutrient-specific influx membrane transporters are unable to transport flavonoids,

while various efflux transporters, which pump drugs and xenobiotics out of cells, recognize flavonoids, hence determining various levels of flavonoid–drug interactions (Passamonti *et al.*, 2009). The transporter protein 2 (MRP2) or the P-glycoprotein have been observed to be responsible for glucuronide extrusion from enterocytes back to the gut lumen (Ayrton and Morgan, 2001), further reducing flavonoid bioavailability. Several *in vitro* investigations have reported effects of flavonoids on enzymes involved in drug metabolism or on membrane transporters involved in drug excretion (Cermak and Wolfram, 2006). Various flavonoids are able to interact with ATP-binding cassette transporters, mainly P-glycoprotein, the multidrug resistance-associated proteins and the breast cancer resistance protein (Brand *et al.*, 2006). These transporters are characterized by loose substrate specificity and are involved in limiting the access of non-nutrient compounds to cells (Passamonti *et al.*, 2009). Moreover, interactions of flavonoids with drug metabolism enzymes, such as the cytochrome P450 (cyp450) system, have been also reported to occur in the small intestine (Moon *et al.*, 2006; Fujita *et al.*, 2008).

Most of the absorbed metabolites are excreted via urine, but it is possible that some are recycled back into the small intestine through the enterohepatic transport system (Donovan *et al.*, 2006). It is believed that extensively conjugated metabolites are eliminated through the bile, with the smaller ones mainly founded in urine (D'Archivio *et al.*, 2007). Although data on bioavailability indicate limited absorption of flavonoids, great variability among different compounds has been observed, depending upon chemical structures (Simons *et al.*, 2010), type of glycosides (Hollman *et al.*, 1995) and food matrix (Serafini and Crozier, 2003). Inter-individual variations have also been observed, probably due to differences in the colonic microflora (Setchell *et al.*, 2002), which can differently affect flavonoid metabolism.

15.3.1 Flavonols

Among the different flavonols, quercetin is the one about which the largest amount of information is available. Its bioavailability is different among food sources, depending on the type of glycosides they contain, with glucosides being much more efficiently and rapidly absorbed than rutosides. Onions, containing quercetin-3-O-glucosides, are among the best sources of bioavailable quercetin, with plasma T_{max} values for the glycoside metabolites of 1–1.5 h and plasma half-lives of around 2.3 h, suggesting absorption at the small intestine level and rapid removal from the circulatory system (Crozier *et al.*, 2009). In the case of the rutoside metabolites, plasma T_{max} have been found to be extended to 5 h, in keeping with large, rather than small, intestinal absorption (Jaganath *et al.*, 2006). Plasma metabolites comprise quercetin-3-O-sulfate, quercetin-3-O-glucuronide, quercetin-O-glucuronide-O-sulfate, isorhamnetin-3-O-glucuronide and quercetin-O-diglucuronide. Differences observed between plasma and urine metabolites are indicative of extensive phase II metabolism, although the sites in the body where these conversions occur have not been yet determined. Urinary

recoveries of these compounds are usually lower than 5% of initial intake (Del Rio *et al.*, 2010).

15.3.2 Flavones

The mechanism of intestinal absorption and the pharmacokinetic of flavones have not been well elucidated so far. Animal studies suggest flavone-glucoside absorption occurs after hydrolysis to free forms by intestinal microbiota (Shimoi *et al.*, 1998; Hanske *et al.*, 2009). Free luteolin and its monoglucuronide in human serum have been detected after ingestion of luteolin, suggesting some luteolin can escape the intestinal conjugation and the hepatic sulfation/methylation systems (Shimoi *et al.*, 1998).

15.3.3 Flavanones

Flavanone glucuronides are found in human plasma 4–5 h after ingestion, in agreement with absorption occurring in the large intestine (Manach *et al.*, 2005). Major metabolites include hesperetin 7-O-glucuronides in plasma and hesperetin/naringenin 7/4'-O-glucuronides, sulfates and diglucuronides in urine, suggesting substantial phase II post-absorption metabolism. Urine concentrations generally account for almost 8% of the initial intake for both naringenin and hesperetin (Mullen *et al.*, 2008). Microflora metabolites of hesperetin have been also detected in human urine, accounting for up to the 37% of hesperetin rutinoside intake (Roowi *et al.*, 2009).

15.3.4 Isoflavones

Isoflavones are the most well absorbed flavonoids, with a C_{max} of almost 2 µmol/l and an average urinary excretion of 42% of initial intake (50 mg intake) for daidzein and 15.6% for genistein (Manach *et al.*, 2005). Absorption takes place in the small intestine although microfloral metabolism is also important, as it forms active metabolites such as equol. Great inter-individual differences in the capability to produce equol by gut microflora have been reported, with only the 30–40% of the western population estimated to be 'equol producers' (Setchell *et al.*, 2002). Nevertheless, a clear dietary influence on the capacity to produce equol has not yet been demonstrated.

15.3.5 Flavanols

Flavan-3-ols are mainly represented by catechins, epicatechins and their galloilated forms from green tea, cocoa and red wine. Epigallocatechingallate (EGCG) is the only flavonoid able to cross the intestine barrier without modifications, and it is found in plasma in a free form. All the other catechins circulate in a conjugated and/or methylated form. Very small C_{max}s, usually in the nanomolar range, are reached at 1–3 h post-ingestion, with half-lives between the 2 and 6 h (Manach *et*

al., 2005; Crozier *et al.*, 2009). These data are in agreement with small intestine absorption, a scarce bioavailability and a rapid turnover of catechins and their parent compounds. The polymeric catechin forms, procyanidins, are not absorbed in the small intestine and reach the large intestine intact, where they are catabolized to phenolic acids by the colonic microflora.

15.3.6 Anthocyanins

Although recent studies report glucuronides and sulfites in human plasma and urine (Felgines *et al.*, 2003; Mullen *et al.*, 2008), glycosides are the predominant circulating anthocyanins forms after ingestion. They are rapidly absorbed and readily eliminated, with T_{max} of around 1.5 h, in keeping with small intestine absorption, and half-lives of about 2.5 h (Manach *et al.*, 2005). Urinary recovery is generally small, most of the time far less than 1% of initial intake, although differences linked to the nature of the aglycones have been reported (Felgines *et al.*, 2003).

15.4 *In vitro* effects of flavonoids on cellular functions

Flavonoids have been suggested to modulate the activity of different cell types involved in both innate and acquired immunity (Fig. 15.2). Oxygen free radicals and non-radical reactive oxygen intermediates, collectively defined as reactive oxygen species (ROS), released by neutrophils and other phagocytes have been increasingly implicated in inflammatory/immune disorders (Middleton *et al.*, 2000). In addition, a 'balance' between the effects of pro-inflammatory – interleukin (IL)-1 β , IL-2, tumor necrosis factor (TNF)- α , IL-6, IL-8 and interferon (IFN)- γ – and anti-inflammatory – IL-10, IL-4 and transforming growth factor (TGF)- β – cytokines is thought to determine the outcome of disease, whether in the short or long term (Santangelo *et al.*, 2007).

Flavonoids may affect different aspect of immune response as described in Fig. 15.2. Flavonoids have been suggested to inhibit oxidative burst and to scavenge ROS, to interfere with the antigen presenting cell (APC) activity of monocytes and dendritic cells (DCs) and to inhibit the release of mediators from mastocytes and granulocytes. The effect of flavonoids on Th1/Th2 cytokine balance suggests that they diminish the Th1-type response. The effect on viability suggests a cytostatic activity on proliferating cells, with increases in activation-induced cell death (AICD) and apoptosis of leukemic cells, although they act as cytoprotective versus ROS-induced cell death.

15.4.1 Effects on oxidative burst

Flavonoids could significantly impair the production of ROS by neutrophils and other phagocytic cells (Fig. 15.2); this may be accompanied by inhibition of NADPH oxidase and myeloperoxidase (Middleton *et al.*, 2000). Flavonoids may

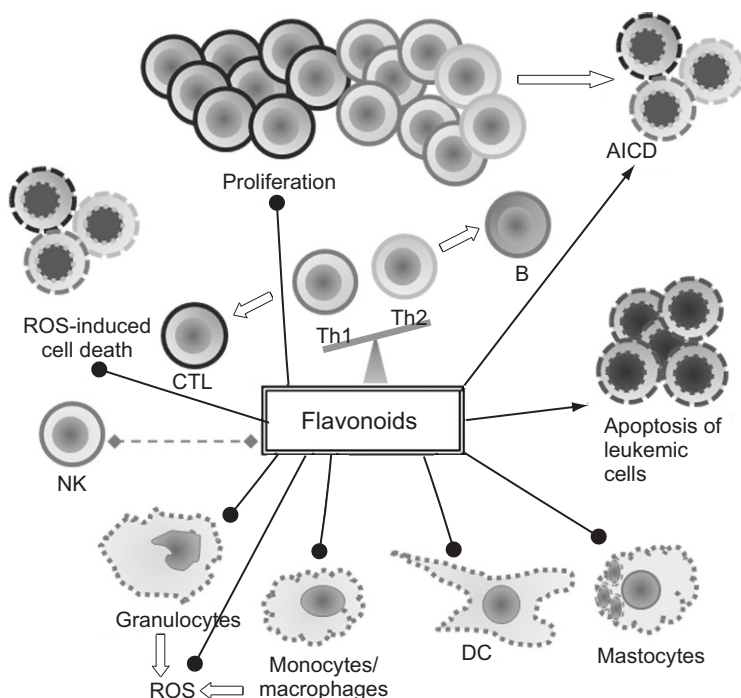


Fig. 15.2 Possible mechanism of action of the immune-modulating activities of flavonoids.

AICD, activation-induced cell death; B, B lymphocytes; CTL, cytotoxic lymphocytes; DC, dendritic cells; NK, natural killer; ROS, reactive oxygen species;

—→, increase; —●, decrease.

also inhibit inducible nitric oxide synthase (iNOS); in particular rutin (20 $\mu\text{g/ml}$) showed a clear inhibition (92% with respect to control) of NO production by lipopolysaccharide (LPS)-treated macrophages (Han, 2009). The aglicone quercetin (100 μM) suppressed ROS production by LPS-stimulated macrophages (Cho *et al.*, 2003; Mamani-Matsuda *et al.*, 2004). Quercetin (40 μM) could also inhibit LPS-induced superoxide production by neutrophils (Liu *et al.*, 2005a). Also various other flavonoids (flavone, daidzein, genistein, isorhamnetin, kaempferol, naringenin and pelargonidin) at a concentration of 100 μM inhibited NO production by more than 50% in LPS-treated J774 macrophages (Hämäläinen, 2007). Epicatechin (50–500 μM) inhibited oxidative burst of activated granulocytes (Sanbongi *et al.*, 1997) and at 200–400 μM inhibited NO secretion by activated macrophages as did a cocoa extract (5–100 $\mu\text{g/ml}$) (Ramiro *et al.*, 2005).

Flavonoid metabolites may exert stronger activity than the parental compounds. In RAW macrophages stimulated with LPS plus IFN- γ , IC₅₀ values for nitrite release were 1.25 mM and 1.25 μM for morin and morin sulfates/glucuronides, respectively (Fang *et al.*, 2003). The metabolite quercetin 3-glucuronide caused a significant reduction in N-formyl-methionyl-leucyl-phenylalanine (fMLP)-evoked calcium influx in human neutrophils, while neither quercetin 3'-sulfate nor querce-

tin produced a similar change (Suri *et al.*, 2008). In addition, the microbial metabolite 3,4-dihydroxyphenylacetic acid, from quercetin glycosides, at 1 μM showed an inhibition of ROS production by human polymorphonuclear leucocytes by 84% when fMLP was used as stimulant (Merfort *et al.*, 1996). Moreover, catechin metabolites inhibited ROS generation in IL-1 β -stimulated human aortic endothelial cells (HAECs) when catechin had no effect (Koga and Meydani, 2001).

15.4.2 Effects on antigen presenting cells (APCs), basophils and mastocytes

Flavonoids might modulate APC activity (Fig. 15.2): quercetin and tangeretin have been shown to depress the expression of class II histocompatibility (DR) antigens in human peripheral blood monocytes (Middleton *et al.*, 2000). Apigenin (20 μM) significantly attenuated the expression of CD80, CD86 and MHC class I and II on the surfaces of CD11c⁺ DCs and impaired the ability of DCs to generate large quantities of IL-12 (Yoon *et al.*, 2006).

Quercetin (0.1–10 $\mu\text{g/ml}$) significantly decreased basophil marker (CD63 and CD203c) expression triggered by stimulation with anti-IgE (Chirumbolo *et al.*, 2010). Quercetin also inhibited antigen-stimulated histamine release by human basophils (Middleton *et al.*, 2000) in a concentration-dependent manner, while the flavonoid glycosides, rutin and naringin, the flavanones, taxifolin and hesperetin, as well as morin, catechin and cyanidin were ineffective (Middleton *et al.*, 2000). Histamine release by antigen-challenged mast cells was significantly inhibited (more than 90% respect to the control) by 160 $\mu\text{g/ml}$ rutin or chlorogenic acid (Chen *et al.*, 2000). The inhibitory effect of flavonoids is not limited to basophils and mast cells; quercetin was able to inhibit stimulated neutrophil lysosomal enzyme release, while rutin and morin were inactive (Middleton *et al.*, 2000).

15.4.3 Effects on Th1/Th2 cytokine balance

It is generally accepted that Th1-derived cytokines (IL-2, IFN- γ and IL-12) promote cellular immunity, while Th2-derived cytokines (IL-4, IL-5 and IL-6) upregulate humoral immunity and exert negative effects on cellular immunity. Production of the Th2 cytokines IL-10 and IL-13, the Th1 cytokine IFN- γ and the inflammatory cytokines TNF- α and IL-6, upon immunological challenge in mast cells, was augmented by rutin (Chen *et al.*, 2000). In contrast, in human umbilical cord blood-derived mast cells, the release of IL-6, IL-8 and TNF- α was inhibited by quercetin, kaempferol, myricetin and morin in a dose-dependent manner (10–100 μM) (Kempuraj *et al.*, 2005). Quercetin (0.1–0.2 mM) caused a decrease in LPS-stimulated release of TNF- α by RAW macrophages (Wadsworth and Koop, 1999) and inhibited (10–80 μM) the secretion of IL-6 by LPS-stimulated neutrophils (Liu *et al.*, 2005b). Luteolin (50 μM) blocked the expression of TNF- α , IL-6 and IL-12 in bone marrow-derived macrophages (Lee *et al.*, 2009). Genistein inhibited IL-1, IL-6 and TNF- α production in LPS-induced human blood monocytes, as well as TNF- α production from LPS-treated RAW cells (Kim *et al.*, 2004) and

apigenin (10 μM) showed significant inhibition of IL-1 β , TNF- α and IL-8 release in monocytes cultured with LPS (Nicholas *et al.*, 2007). Epicatechin (10–200 μM) and isoquercitrin (5 and 40 μM) significantly reduced TNF- α secretion (Ramiro *et al.*, 2005). Quercetin and catechins coupled their inhibitory action on TNF- α with the increase of IL-10 release (Santangelo *et al.*, 2007).

Flavonols were able to decrease production of the Th1 cytokine IL-2: apigenin, luteolin and genistein completely prevented the concanavalin-A (Con-A)-elicited increase of IL-2 (Lopez-Posadas *et al.*, 2008). In agreement with these findings, luteolin (1–100 μM) inhibited IL-2 release from Jurkat cells, whether activated by anti-CD3/anti-CD28 or by mast cells (Kempuraj *et al.*, 2008). Also pre-treatment with EGCG (50–100 μM) inhibited *Staphylococcus aureus* enterotoxin B (SEB)-induced IL-2 secretion by peripheral blood mononuclear cells (PBMCs) (Watson *et al.*, 2005). Kaempferol (100 μM) lowered IFN- γ concentration in whole blood cultures but the concentrations of IL-2 and of the Th2 cytokine IL-4 were unaffected (Miles *et al.*, 2005). In contrast, Nair *et al.* (2002a) reported that quercetin (0.5–50 μM) significantly induced the gene expression as well as the production of Th1-derived IFN- γ and downregulated Th-2 derived IL-4 by PBMCs. Quercetin at concentrations ranging between 1 and 50 μM showed a dose-dependent decrease in TNF- α gene expression in PBMCs (Nair *et al.*, 2006).

In whole blood cultures, Crouvezier *et al.* (2001) showed no significant effect of epicatechin or epicatechingallate, up to a concentration of 20 μM , on the production of IL-1 β , IL-6 or TNF- α . Epigallocatechin and EGCG significantly inhibited the production of IL-1 β at 10 and 20 μM , but had no effect on the production of IL-6 or TNF- α . Epicatechin had no effect on the production of IL-10 in human whole blood cultures, whereas epicatechin gallate, epigallocatechin and EGCG significantly enhanced production of IL-10 at 10 μM and above. In the same study, no effect of epicatechin on the production of IL-1 β , IL-6 or TNF- α by PBMCs was shown. Epicatechin gallate and epigallocatechin significantly inhibited the production of IL-1 β at 20 μM , but had no effect on the production of IL-6 or TNF- α . EGCG at 10 and 20 μM strongly inhibited the production of IL-1 β and, unlike its effects in whole blood cultures, also inhibited the production of IL-6 at these concentrations; however, EGCG did not have any effect on TNF- α production (Crouvezier *et al.*, 2001).

When food extracts were tested, cocoa (50 $\mu\text{g}/\text{mL}$) significantly decreased TNF- α secretion (Ramiro *et al.*, 2005). IL-2 and IgG production by cultured human PBMCs were both inhibited by the addition of cocoa liquor containing polyphenols in a dose-dependent manner in the range of concentration between 25 and 100 $\mu\text{g}/\text{ml}$ (Sanbongi *et al.*, 1997). More recently (Ramiro-Puig and Castell, 2009), cocoa extract has been found to slightly increase *in vitro* IL-4 secretion and, therefore, to favour the Th2 response. Both black and green tea extracts caused a significant concentration-dependent decrease of LPS-induced IL-1 β , IL-6, IL-8 and TNF- α secretion by human monocytes (Pajonk *et al.*, 2006). Grape seed extract (0.1–0.5 mg/ml) (Nair *et al.*, 2002b), containing procyanidins, induced the production of the Th1-derived cytokine IFN- γ by unstimulated PBMCs, but had no effect on the Th2-derived IL-6 production.

The degree of polymerization of flavonoids may affect their anti-inflammatory activity (Ramiro-Puig and Castell, 2009). When PBMCs were co-incubated with phytohemagglutinin (PHA) and different cocoa fractions (25 µg/ml), the small oligomers (monomer through tetramer) suppressed PHA-induced expression and/or secretion of IL-1β, while the larger oligomers (hexamer through decamer) continually augmented IL-1β (Mao *et al.*, 2000a). Mao *et al.* (2000b) showed that the pentamer, hexamer and heptamer (at 50 µg/ml) effectively inhibited IL-2 gene expression in PHA-stimulated cells, while the dimeric, the trimeric, hexameric and decameric procyanidin fractions inhibited PHA-induced expression of IL-4. The intermediate-sized fractions (tetramers through octamers; 25 mg/ml) significantly enhanced TNF-α secretion in PBMCs (Mao *et al.*, 2002). Also, in the study conducted by Kenny *et al.* (2007) PBMCs were cultured in the presence of cocoa flavanol fractions (20 µg/ml) at a different degree of flavanol polymerization; the chain length of flavanol fractions had a significant effect on cytokine release from both unstimulated and LPS-stimulated PBMCs. There was a striking increase of LPS-induced synthesis of IL-1β, IL-6, IL-10 and TNF-α in the presence of long-chain flavanol fraction (hexamers to decamers) compared with short chain flavanol fraction (monomers to pentamers). The effects of cocoa flavonoids on cytokine secretion seem to be related to the degree of polymerization: short-chain cocoa procyanidins increase IL-4 and IL-5, whereas long-chain procyanidins reduce production of both these Th2 cytokines (Ramiro-Puig *et al.*, 2009). Following from that, the effects on the balance between Th1 and Th2 cytokine expression seems to be specific for different cytokines and influenced by flavonoid structures highlighting the difficulties in understanding the mechanism of their action (Fig. 15.2).

The only study utilizing flavonoid metabolites, conducted by Monagas *et al.* (2009), investigated the effect of microbial phenolic metabolites of proanthocyanidins (1 µM); the dihydroxylated phenolic acids, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid caused a decrease in the secreted levels of TNF-α, IL-1β and IL-6 from LPS-stimulated PBMCs. Monohydroxylated phenolic acids did not produce significant changes in cytokine secretion with the exception of 4-hydroxyhippuric acid on TNF-α secretion, which produced a significant increase in this cytokine.

15.4.4 Effects on proliferation and cell death

Lymphocyte proliferation and AICD, fundamental physiological responses involved in clonal expansion after antigen challenge, could both be influenced by flavonoid exposure. Modulation of cytokines as described in the previous section, in particular a reduction in production of the Th1 cytokine IL-2, may affect both antigen-specific and polyclonal proliferative responses (Fig. 15.2).

Kaempferol prevented proliferation in the mixed lymphocyte culture (MLC) *in vitro* test measuring allogeneic reactivity between lymphocytes (Cortes *et al.*, 2007), in a dose-dependent manner (10–40 µM). In phorbol myristate acetate (PMA)-stimulated lymphocytes and in PHA-stimulated PBMCs, cocoa extract

reduced lymphocyte proliferation by downregulating IL-2 secretion and IL-2 receptor expression (Ramiro-Puig and Castell, 2009). EGCG (50–100 μM) inhibited Con A- and SEB-induced proliferation by PBMCs, while epigallocatechin (100 μM) reduced the proliferative response to SEB and Con A, but it was significantly weaker than eEGCG (Watson *et al.*, 2005). In the presence of the flavones apigenin and luteolin (3.5–35 μM), the antigen-specific proliferative response of PBMCs against the myelin protein α -B-crystallin was reduced by 16% and 23%, respectively. In contrast, the flavonols morin and fustin did not exert any inhibitory effects while the flavonol quercetin and the flavanone hesperitin were effective only at the highest dose tested (35 μM) (Verbeek *et al.*, 2004). Apigenin, genistein, luteolin and quercetin (50 μM) showed a small but significant decrease in lymphocyte viability (Lopez-Posadas *et al.*, 2008). Quercetin (10–100 μM) inhibited PHA-induced proliferation of PBMCs, but altered viability only at concentration of 100 μM , while at 50 μM there was an induction of apoptosis in leukemic cell lines (Lugli *et al.*, 2009). Rutin at a concentration of 40 $\mu\text{g/ml}$, decreased T-cell proliferation in response to Con A (Han, 2009). A decrease in cell proliferation was found for isolated blood lymphocyte cultures treated with 2.5, 5 and 10 $\mu\text{g/ml}$ apigenin (Rithidech *et al.*, 2005). Apigenin (12.5–75 μM) induced significant apoptosis in a human CD4 T-cell line and in Jurkat T cells, in a dose- and time-dependent manner and increased AICD in human CD4 T cells (Xu *et al.*, 2008). By contrast, apigenin did not increase anti-CD3-induced apoptosis in stimulated fresh human CD4 T cells. In terms of mechanism of action, Lee (1995) clearly demonstrated that, for significant suppression of lymphocyte proliferation *in vitro*, O-methyl group at C-4' is essential and one more methyl substitution at either C-7 or C-4 increases the suppressive activity.

Cytostatic effects of flavonoids on malignant leucocyte-derived cells have also been observed (Fig. 15.2). In human B malignant cells, genistein decreased viability in a dose-dependent manner (7.5–60 μM) with maximal effect at 30 μM (Mansour *et al.*, 2004). Antiproliferative activity of epigallocatechin on human lymphoblastoid leukemia lymphocytes has an IC₅₀ of 0.4 μM (Lu *et al.*, 1998). Cytostatic effects of tangeretin on HL-60 cells were dose-dependent with an IC₅₀ of 0.173 μM , without any effect on cell viability even at 27 μM (Hirano *et al.*, 1995). However, tangeretin showed almost no effect on growth of the human T-lymphocytic leukemia cell line MOLT-4 (IC₅₀ of 13.0 μM) and on human PBMCs stimulated with Con A (IC₅₀ of 10.0 μM) (Hirano *et al.*, 1995).

Flavonoids may exert cytoprotective effects through their ability to limit damage induced by exogenously induced ROS (Fig. 15.2). Pure quercetin and grape-derived beverages restored proliferation in the presence of minimally modified low-density lipoprotein (MM-LDL) and prevented the apoptosis otherwise induced by lovastatin (Carrero *et al.*, 1998). Phenolic components of wine are protective against the DNA-damaging and cytotoxic effects of hydrogen peroxide and γ -radiation *in vitro* (Greenrod and Fenech, 2003). Hesperidin at doses of 10, 50 and 100 μM protected whole blood samples against genotoxicity induced by radiopharmaceuticals (Hosseini-mehr *et al.*, 2009). Quercetin itself induced chromosomal damage at 10 μM , but quercetin-3-O-sulfate and isorhamnetin did not

induce any damage up to 30 μM (Saito *et al.*, 2004). On the other hand, pretreatment with quercetin (1 μM), quercetin-3-O-sulfate (10 μM) or isorhamnetin (5 μM) prevented H_2O_2 -induced chromosomal damage (Saito *et al.*, 2004).

15.5 Molecular mechanisms

ROS, generated by phagocytes during innate immune responses, play a key role in enhancing inflammation through the activation of NF κ B and AP-1 (Rahman *et al.*, 2006; Gopalakrishnan and Kong, 2008). Antioxidants inhibiting Th1-type immune activation, may promote Th2-type immunity; in contrast, ROS may promote cellular cytotoxicity by induction of Th1-type cytokines like IFN- γ (Murr *et al.*, 2005). Thus, an integrated effect on redox-modulated signaling pathways results in complex effects on immune function. The molecular mechanisms involved in the anti-inflammatory activities of flavonoids have been suggested to include: induction of Nrf2, activation of phase II antioxidant detoxifying enzymes, inhibition of pro-inflammatory enzymes, such as cyclooxygenase (COX), lipoxygenase (LOX) and iNOS, inhibition of NF κ B and AP-1 pathways, modulation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activity (Serafini *et al.*, 2010).

Among the major classes of transcription factors (TFs) involved in flavonoid action on immune cells, Nrf2 is a member of the 'cap'n collar' (CNC-bZIP subfamily of basic/leucine zipper (bZIP)) family (Mann *et al.*, 2007a,b). These TFs bind to the nuclear factor erythroid-derived 2 (NF-E2)-binding sites that are essential for the regulation of erythroid-specific genes. NF-E2-related factor 2 is a redox-sensitive factor whose nuclear translocation and binding to the antioxidant response elements (AREs) in gene promoter regions may result in induction of antioxidant enzymes. Nrf2 is expressed in a wide range of tissues, many of which are sites of expression for phase II detoxifying genes that are inducible by xenobiotics and antioxidants. The body of evidence suggests that dietary flavonoids such as quercetin, apigenin, resveratrol, curcumin, catechins and gallic esters can modulate inflammatory responses also through an activation of pathways inducing antioxidant transcription and detoxification defense systems, such as glutathione peroxidase, heme oxygenases, γ -glutamylcysteine synthetase, superoxide dismutase and glutathione reductase, through AREs (Masella *et al.*, 2005; Rahman *et al.*, 2006; Mann *et al.*, 2007a,b). Green tea polyphenol extract, and EGCG in particular, stimulates the transcription of phase II detoxifying enzymes through AREs (Yu *et al.*, 1997, 2000; McKay and Blumberg, 2002).

NF κ B is one of the main inducible transcription factors whose modulation triggers a cascade of molecular events, some of which can constitute potential key targets for the treatment of inflammation (González-Gallego *et al.*, 2007). Oxidative stress is an important stimulus for the activation of NF κ B which appears in latent form in the cytoplasm of non-stimulated cells, forming a complex with its inhibitors I κ Bs (α and β). When the cell is stimulated, NF κ B is activated by means of the phosphorylation and degradation of I κ B proteins and migrates to the nucleus

stimulating the expression of its target genes. Activation of NF κ B mediates a coordinated regulation of target genes, followed by secretion of pro-inflammatory signaling molecules such as acute-phase proteins, chemokines and cytokines, adhesion molecules, growth factors and inducible enzymes (González-Gallego *et al.*, 2007). Another well-characterized transcription factor related to oxidative stress, AP-1, is composed of homodimers and/or heterodimers of bZIP domain proteins, including Jun, Fos, Maf and ATF subfamilies, and it regulates the transcription of various genes associated with cellular inflammation, proliferation and apoptosis (Gopalakrishnan and Kong, 2008). The intracellular levels of c-jun and c-fos are controlled mainly by their transcription rates, which are tightly regulated by a variety of stimuli. AP-1 activation is regulated at both transcript and protein levels.

Effects of flavonoids on the binding capacity of NF κ B and AP-1 may be regulated through the inhibition of protein kinase involved in signal transduction such as PKC and MAPK, a family of serine/threonine protein kinase that mediate fundamental biological processes and cellular responses to external stress signal (Kaminska, 2005). Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and by modulating gene expression. In response to inflammatory stimuli, intracellular signaling pathways in macrophages are activated, leading to the production of inflammatory mediators. In macrophages and other cell types, LPS activates three kinds of MAPs: extracellular signal-related kinase (ERK); JUN N-terminal kinase (JNK) and p38 kinase. Changes in the cellular redox status may result in the activation of pro-apoptotic signaling proteins such as JNK which may initiate apoptotic mechanisms within cells (Santangelo *et al.*, 2007). Flavonoids can reduce the phosphorylation and degradation of I κ Ks inhibiting the activation of NF κ B and the production of pro-inflammatory signaling molecules and a series of enzymes which are activated in the course of the inflammatory process: iNOS, COX-2, LOX, C-reactive protein, cytokines and adhesion molecules.

Quercetin (50–200 μ M) was shown to affect NF κ B activation by ERK and p38 kinase inhibition in LPS-stimulated RAW 264.7 cells (Cho *et al.*, 2003) and, at lower concentrations (5–50 μ M) down-regulated TNF- α protein expression by PMBCs (Nair *et al.*, 2006). Also apigenin (10 μ M) inhibited via NF κ B pathway the production of the pro-inflammatory cytokines IL-1 β , IL-8 and TNF α in LPS-stimulated human monocytes (Nicholas *et al.*, 2007). Fisetin (30 μ M) significantly decreased the PMA plus calcium ionophore A23187 (PMACI)-induced secretion of TNF- α , IL-1 β , IL-4, IL-6 and IL-8 in human mast cells (Park *et al.*, 2007). Quercetin and rutin, at the same concentration, decreased gene expression and production of all the pro-inflammatory cytokines after human mast cell stimulation with PMACI; myricetin-attenuated TNF- α and IL 6 but not IL-1 β and IL-8 with astragalin and kaempferol having no effect. These findings suggest that fisetin and quercetin attenuate production of pro-inflammatory cytokines by reducing the activation of NF κ B. In contrast, rutin reduces the expression of pro-inflammatory cytokines without affecting NF κ B transcription, but acts via other transcription factors or repressor proteins (Park *et al.*, 2008). Quercetin, naringenin and

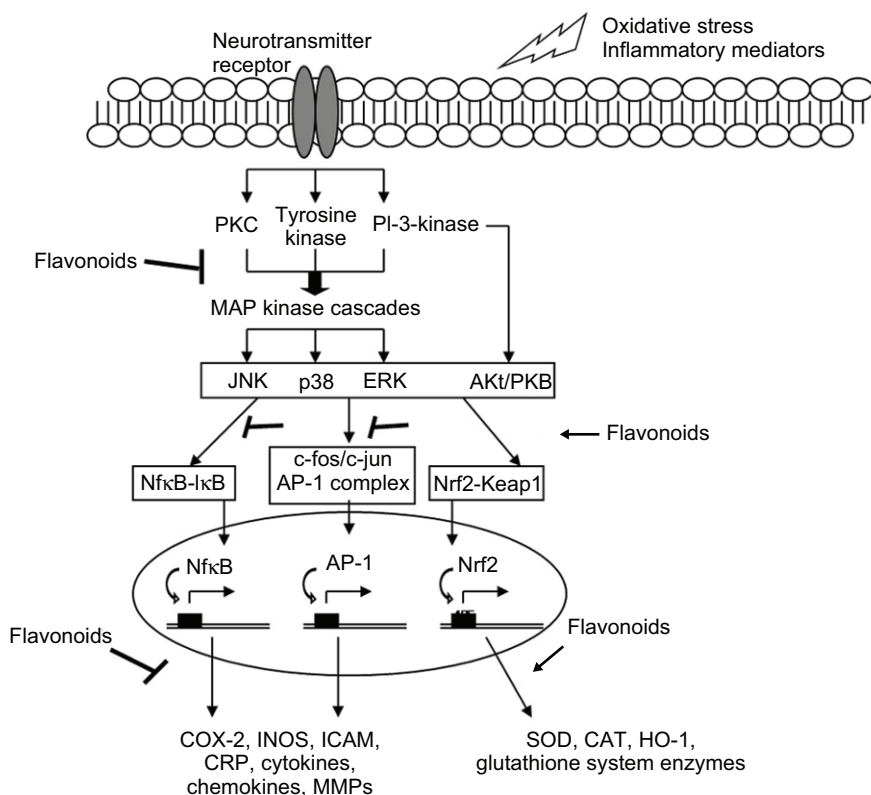


Fig. 15.3 Flavonoid modulation of selected signal transduction pathways involved in immune function. Akt/PKB, protein kinase B; AP-1, activated protein-1; CAT, catalase; COX-2, cyclooxygenase-2; CRP, C-reactive protein; ERK, extracellular signal-related kinase; JNK, Jun N-terminal kinase; HO-1, heme-oxygenase-1; ICAM, intercellular adhesion molecule; IκB, inhibitory protein of nuclear factor κB; iNOS, inducible nitric oxide synthase; MAP, mitogen-activated protein; MMPs, metalloproteases; NFκB, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2-related factor; PKC, protein kinase C; SOD, superoxide dismutase.

pelargonidin (100 μM) inhibited the LPS-induced activation of NFκB by more than 80% in J744 cells, while flavone, genistein, isohamnetin, kaempferol and daidzein (100 μM) had a weaker inhibitory effect (Hämäläinen *et al.*, 2007). EGCG (100–200 μM) prevented the expression of COX-2 protein in human chondrocytes (Ahmed *et al.*, 2002) and IL-12 production, by inhibiting phosphorylation of p38 MAPK, augmenting phosphorylation of ERK and nuclear protein-binding to NFκB site (Yoon and Back, 2005; Santangelo *et al.*, 2007). Curcumin and epigallocatechin-3-gallate upregulated expression of antioxidant genes, such as superoxide dismutase, glutathione peroxidase and catalase, through modulation of MAPK-ARE-Nrf2 pathway (Fig. 15.3).

Flavonoid anti-inflammatory activity might take place also through interaction with lipid rafts implicated in signaling from the T-cell antigen receptor (TCR)

(Kabouridis and Jury, 2008). Membrane lipid rafts, which are sphingolipid- and cholesterol-enriched membrane microdomains, assemble important signaling proteins into complexes prone to be activated by molecular triggers (Brown, 2006; Sengupta *et al.*, 2007). It has been demonstrated that the lipid raft is used as a platform by 67 kDa laminin receptor (LamR). LamR may systematically reshape the rafts and affect uptake of EGCG (Patra *et al.*, 2008). This flavonoid will first be incorporated into the plasma membrane, and is then re-loaded into the rafts where LamRs are present. In the rafts, EGCG interacts with many receptors destabilizing rafts structure and inactivating MAPK signaling. The structure–activity relationship analysis of major catechins on cell surface-binding suggested that binding activities of pyrogallol-type catechins (EGCG6 and gallocatechingallate) were higher than those of catechol-type catechins (epicatechingallate and catechingallate) (Patra *et al.*, 2008).

15.6 Human intervention studies with flavonoids

Dietary intervention trials investigating the effect of flavonoids on circulating cytokine levels in humans are scarce and are focused on a limited number of foods of plant origin, as described in Table 15.2. Of the 22 studies present in literature, 10 used soy, five fruit juices, three teas, two red wines and two vegetable soups, with a flavonoid intake ranging from 70–160 mg/day. Only six of these studies assessed polyphenol bioavailability: plasma levels ranged between 12 nM and 150 nM, while urinary levels ranged from 5–18 μ M (Table 15.2).

In diabetic subjects, IL-6 and TNF- α were unaffected by a daily intake of three cups of black tea, although the flavonoid metabolite 4-O-methylgallic acid was excreted in urine (Mukamal *et al.*, 2007). Similar results were obtained after consumption of green tea (900 mL) on circulating levels of IL-6 in diabetic patients (Ryu *et al.*, 2006). Four-week administration of black and green tea had no effect on plasma IL-6, IL-1 β and TNF- α in healthy subjects (de Maat *et al.*, 2000).

Studies with fruit juices, vegetable soups and red wine are extremely contradictory (Table 15.2). Udani *et al.* (2009) reported a dose–response decrease in IL-12 levels after three different doses of mangosteen juice. Bilberry juice consumption (Karlsen *et al.*, 2010) decreased IL-6 and IL-15 levels and increased TNF- α , concurrently with increases of plasma quercetin and p-coumaric acid concentration. In contrast, Boots *et al.* (2008) showed no effect on TNF- α after four-weeks consumption of blueberry and apple juice despite an increase in plasma quercetin. Also plasma IL-6 levels were unaffected by orange and blackcurrant juice consumption in peripheral arterial disease patients (Dalgård *et al.*, 2009). In contrast, tropical camu-juice reduced both IL-6 and IL-8 in smokers (Inoue *et al.*, 2008). Sánchez-Moreno *et al.* in two consecutive studies (2004, 2006) reported no effect on TNF- α , IL-1 β and IL-6 levels after two weeks consumption of a Mediterranean vegetable soup. One year of red wine consumption decreased TNF- α , IL-6 and IL-18 in diabetic patients more than a Mediterranean diet alone (Marfella *et al.*, 2006). Estruch and coworkers (2004) found reduced levels of IL-1 α after four-weeks of

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Table 15.2 Overview of human chronic intervention studies on the effects of flavonoids-rich food and beverages on circulating cytokine concentrations

Treatment (daily dose)	Days	Subjects	Study design	n	Effect on cytokines	Effect on phenolics	References
Black tea (1 cup, 318 mg/day catechins)	180	Diabetics	Placebo-controlled parallel	14 × 2	TNF- α and IL-6 $\leftrightarrow$	Urinary 4-O-methylgallic acid $\uparrow$ (13 μ M)	Mukamal <i>et al.</i> , 2007
Black tea, green tea (900 ml)	28	Healthy	Placebo-controlled parallel	16 black tea, 15 green tea, 15 control	TNF- α , IL-1 β and IL-6 $\leftrightarrow$		de Maat <i>et al.</i> , 2000
Green tea (900 ml)	28	Diabetics	Placebo-controlled crossover	55	IL-6 $\leftrightarrow$		Ryu <i>et al.</i> , 2006
Juice (bilberry) (330 ml)	28	Healthy	Placebo-controlled parallel	31 × 2	IL-6 and IL-15 $\downarrow$, TNF- α $\uparrow$, IFN- γ , IL-17, IL-13, IL-12, IL-8, IL-7, IL-5, IL-4, IL-2 and IL-1 $\leftrightarrow$	Plasma quercetin $\uparrow$ (12 nM) and p-coumaric acid $\uparrow$ (8.7 nM)	Karlsen <i>et al.</i> , 2010
Juice (non-specified volume of blueberry–apple providing 97 mg/l quercetin)	28	Healthy	Uncontrolled	7	TNF- α $\leftrightarrow$	Plasma quercetin $\uparrow$ (50 nM)	Boots <i>et al.</i> , 2008
Juice (fruit tropical camu, 70 ml) or vitamin C (1050 mg)	7	Smokers	Uncontrolled	10 × 2	Camu juice: IL-6 $\downarrow$, IL-8 $\downarrow$		Inoue <i>et al.</i> , 2008
Juice (mangosteen, 180, 360 or 540 ml)	56	Healthy	Placebo-controlled parallel	10 × 4	IL-12 $\downarrow$ with all doses (dose-dependent effect)		Udani, 2009

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Juice (orange and blackcurrant, 500 ml)	28	Peripheral arterial disease	Placebo-controlled crossover	24 juice, 23 control	IL-6 ↔		Dalgard <i>et al.</i> , 2009
Mediterranean vegetable soup (500 ml)	14	Healthy	Uncontrolled	12	TNF- α , IL-1 β and IL-6 ↔		Sánchez-Moreno <i>et al.</i> , 2004
Mediterranean diet ± red wine (118 ml)	365	Diabetics	Placebo-controlled parallel	57 red wine, 58 control	Mediterranean diet ± red wine: TNF- α , IL-6 and IL-18 ↓ (bigger effect when red wine included)		Marfella <i>et al.</i> , 2006
Red wine (320 ml) or gin (100 ml)	28	Healthy	Placebo-controlled crossover	80	↓ IL-1 α (both red wine and gin) TNF- α ↔	Plasma epicatechin gallate ↑ (0.024 mg/L with red wine)	Estruch <i>et al.</i> , 2004
Soy (diet providing 70 mg isoflavones)	28	Hypercholesterolemic	Uncontrolled		IL-6 ↑ (in women) TNF- α ↔		Jenkins <i>et al.</i> , 2002
Soy (protein isolate 25 g providing 90 mg isoflavones)	42	Healthy	Placebo-controlled crossover	64	IL-6 ↔		Hilpert <i>et al.</i> , 2005
Soy (milk providing 112.1 mg isoflavones)	112	Healthy postmenopausal	Uncontrolled	20	IL-6 ↑, TNF- α and IL-1 α ↓		Huang <i>et al.</i> , 2005
Soy (milk 706 mL providing 71.6 mg isoflavones)	112	Healthy postmenopausal	Placebo-controlled	18 soy, 19 control	IFN- γ , IL-2, TNF- α ↔	Plasma: daidzein ↔; genistein ↑ (0.15 μ M); equol ↑ (0.1 μ M) Urine: daidzein ↑ (13 μ M); genistein ↑ (5 μ M); equol ↑ (18 μ M);	Ryan-Borchers <i>et al.</i> , 2006

(Continued)

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Table 15.2 Continued

Treatment (daily dose)	Days	Subjects	Study design	<i>n</i>	Effect on cytokines	Effect on phenolics	References
Soy (protein or nuts 30 g)	56	Postmenopausal with metabolic syndrome	Placebo-controlled crossover	42	TNF- α $\downarrow$ (nuts) IL-18 $\downarrow$ (protein) IL-6 and IL-2 $\leftrightarrow$	Plasma phytoestrogen $\uparrow$ (soy nut 64%; soy protein 48%)	Azadbakht <i>et al.</i> , 2007
Soy (protein 25 g providing 101 mg isoflavones)	56	Healthy postmenopausal	Placebo-controlled crossover	60	IL-6 $\leftrightarrow$		Nasca, 2008
Soy (protein 20 g providing 160 mg isoflavones)	84	Healthy postmenopausal	Placebo-controlled parallel	32 soy, 43 control	TNF- α and IL-6 $\leftrightarrow$		Charles, 2009
Soy (soymilk, 3 cups)	28	Healthy	Placebo-controlled parallel	16 soy, 15 control	TNF- α , IL-1 β and IL-6 $\leftrightarrow$		Beavers <i>et al.</i> , 2009
Soy (100 mg of isoflavones)	730	Healthy	Placebo-controlled parallel	90 soy, 93 control	IL-6 $\leftrightarrow$		Maskarinec <i>et al.</i> , 2009
Soy (protein 10 g)	28	Overweight/obese	Uncontrolled	20	TNF- α $\uparrow$ (overweight and obese) IL-6 $\uparrow$ (obese)		Zemel <i>et al.</i> , 2010

$\leftrightarrow$ No change; $\uparrow$ increase; $\downarrow$ decrease.

Uncontrolled: longitudinal, repeated-measures study, study in which blood was drawn before, defined as baseline (day 0), and after treatment.

red wine consumption; however, the same effect was found also with gin but epicatechingallate concentration increased only after red wine consumption (Table 15.2).

Due to estrogenic activities (Setchell *et al.*, 2002) the majority of long-term intervention studies using soy as source of bioactive molecules have been conducted in postmenopausal women (Table 15.2). These studies found no changes in TNF- α (Jenkins *et al.*, 2002; Hermansen *et al.*, 2005; Ryan-Borchers *et al.*, 2006; Beavers *et al.*, 2009; Charles *et al.*, 2009) and IL-6 levels (Hilpert *et al.*, 2005; Nasca *et al.*, 2008; Beavers *et al.*, 2009; Charles *et al.*, 2009; Maskarinec *et al.*, 2009;). Huang *et al.* (2005) described an increase in circulating levels of IL-6 and a decrease of TNF- α and IL-1 α after soymilk consumption. In contrast, Jenkins *et al.* (2002) did not describe any effect on TNF- α but only on IL-6. Recently, Zemel *et al.* (2010), providing 10 g of soy protein daily, reported increased levels of TNF- α in both obese and overweight subjects and increased levels of IL-6 in obese only. Increase in total isoflavones (Azadbakht *et al.*, 2007) and genistein levels (Ryan-Borchers *et al.*, 2006) induced by long-term soy consumption was associated with cytokine reductions (Azadbakht *et al.*, 2007) or lack of effect (Ryan-Borchers *et al.*, 2006), indicating the difficulty in finding a clear association between effect on inflammatory markers related to increases in flavonoid intake and concentrations.

Intervention trials conducted with flavonoid galenic preparations ($n=10$) or food extracts containing flavonoids ($n=4$) are described in Table 15.3. The inflammatory cytokines IL-8 (Nieman *et al.*, 2007a,b), IL-6 (Nieman *et al.*, 2007a,b; Bae *et al.*, 2009; Charles *et al.*, 2009) and TNF- α (Nieman *et al.*, 2007a,b; Bae *et al.*, 2009; Egert *et al.*, 2009; Heinz *et al.*, 2010) were unaffected by quercetin supplementation, either alone or in combination with vitamin C (Bae *et al.*, 2009), in healthy subjects or in patients with arthritis. In agreement with previous data on food flavonoids (Boots *et al.*, 2008), the absorption of quercetin was not related to any reduction in circulating cytokines (Nieman *et al.*, 2007a,b; Egert *et al.*, 2009; Heinz *et al.*, 2010).

The only study measuring Th2 cytokines after isoquercitin supplementation did not find any changes in IL-4 and IL-5 concentration or in quercetin levels (Kawai *et al.*, 2009). Grape extract (Zern *et al.*, 2005) showed a reduction in TNF- α in both pre- and postmenopausal women but had no effect on IL-6 (Zern *et al.*, 2005). An effect on TNF- α was also seen with a daily supplementation of 455 mg of catechins for seven months in hemodialysis patients (Hsu *et al.*, 2007). However, a shorter supplementation period (28 days) with green tea extract containing catechins (2.49 g/day) was ineffective on both IL-6 and TNF- α levels (de Matt *et al.*, 2000). Six months of supplementation with chokeberry flavonoid extract, resulted in a decrease of plasma IL-6 levels in subjects after myocardial infarction (Naruszewicz *et al.*, 2007). Despite the increase in plasma and urinary levels of genistein and equol, no effect of soy supplements on circulating Th1 cytokines was shown in postmenopausal women (Ryan-Borchers *et al.*, 2006) or in hypercholesterolaemic subjects (Hermansen *et al.*, 2005). Patients with liver cirrhosis showed an elevated serum level of TNF- α (about 4 pg/ml) versus healthy controls, and long-term consumption of a fermented papaya preparation significantly lowered these values (by about 2 pg/ml) (Marotta *et al.*, 2007).

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Table 15.3 Overview of human intervention studies on the effects of flavonoids and food extracts on circulating cytokine concentrations

Treatment (daily dose)	Days	Subjects	Study design	n	Effect on cytokines	Effect on phenolics	References
Catechins (455 mg)	215	Hemodialysis patients	Placebo-controlled parallel	14 catechins 30 control	TNF- α ↓		Hsu <i>et al.</i> , 2007
Chokeberry extract (255 mg)	183	Post-myocardial infarction	Placebo-controlled parallel	22 × 2	IL-6		Naruszewicz <i>et al.</i> , 2007
Green tea extract (3.6 g providing catechins 2.49 g)	28	Healthy	Placebo-controlled parallel	13 green tea 15 control	TNF- α , IL-1 β and IL-6 ↔		de Maat <i>et al.</i> , 2000
Grape extract (36 g providing total phenols 5.8 g/kg)	28	Healthy	Placebo-controlled crossover	44	TNF- α ↓, IL-6 ↔		Zern <i>et al.</i> , 2005
Fermented papaya preparation (9 g)	182	HCV-related cirrhosis or healthy	Placebo-controlled parallel	25 papaya, 25 control (cirrhosis); 10 control (healthy)	TNF- α ↓		Marotta <i>et al.</i> , 2007
Isoquercetin (200 mg)	56	Japanese cedar pollinosis	Placebo-controlled parallel	10 × 2	IL-4, IL-5, IL-12, IL-13 and IFN- γ ↔; IgE ↔	Plasma quercetin ↔	Kawai <i>et al.</i> , 2009
Quercetin (1 g)	21	Healthy	Placebo-controlled parallel	Quercetin 18, control 21	TNF- α , IL-1, IL-6, IL-8 and IL-10 ↔	Plasma quercetin ↑ (500 μ g/l)	Nieman <i>et al.</i> , 2007a

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Quercetin (150 mg)	42	Healthy	Placebo-controlled crossover	93 × 2	TNF- α ↓	Plasma quercetin ↑ (200 nM)	Egert <i>et al.</i> , 2009
Quercetin (1 g)	21	Healthy	Placebo-controlled parallel	20 × 2	TNF- α , IL-1, IL-6, IL-8 and IL-10 ↔	Plasma quercetin ↑ (1000 µg/l)	Nieman <i>et al.</i> , 2007b
Quercetin (50, 100, 150 mg)	14	Healthy	Uncontrolled	11, 12, 12	TNF- α ↔	Plasma quercetin ↑ (92.5, 172.1, 315.8 nM), isorhamnetin ↑ (5.2, 11.8, 22.2 nM)	Egert <i>et al.</i> , 2008
Quercetin (498 mg) + vitamin C (399 mg)	28	Rheumatoid arthritis	Placebo-controlled crossover	20	TNF- α , IL-1 β and IL-6 ↔		Bae <i>et al.</i> , 2009
Quercetin (500 mg, 1000 mg)	84	Healthy	Placebo-controlled parallel	38 quercetin 500 mg, 40 quercetin 1000 mg, 42 control	TNF- α and IL-6 ↔	Plasma quercetin ↑ (300, 500 µg/l)	Heinz <i>et al.</i> , 2010
Soy (100 mg isoflavones)	168	Hyper-cholesterolaemic	Placebo-controlled parallel	43 soy, 46 control	TNF- α ↔		Hermansen <i>et al.</i> , 2005
Soy (70 mg isoflavones)	112	Healthy postmenopausal	Placebo-controlled parallel	19 soy, 15 control	IFN- γ , IL-2 and TNF- α ↔	Plasma: daidzein ↔; genistein ↑ (0.35 µM); equol ↑ (0.03 µM); Urine: daidzein ↑ (24 µM); genistein ↑ (18 µM); equol ↑ (10 µM)	Ryan-Borchers <i>et al.</i> , 2006

HCV, hepatitis C virus.

↔ No change; ↑ increase; ↓ decrease.

Uncontrolled: longitudinal, repeated-measures study, study in which blood was drawn before, defined as baseline (day 0), and after treatment.

Markers of immune function, such as lymphocyte populations, *ex vivo* induced cytokine production, phagocytic activity, lymphocyte proliferation and lytic activity of natural killer (NK) cells have also been investigated for their response to flavonoid supplementation. Acute (Watzl *et al.*, 2002) or chronic (14 days, Watzl *et al.*, 2004) intake of 500 mL of red wine had no effect on *ex vivo* phagocytic activity, lymphocyte proliferation, lytic activity of NK cells and cytokine production in healthy men. Similar results were obtained with a large amount of fruit and vegetables (eight servings/day) for four weeks (Watzl *et al.*, 2005). Long-term (42 days) consumption of dark chocolate (36.9 g/day) and cocoa powder drink (30.95 g/day) did not affect LPS-activated whole blood production of IL-1 β , IL-6 and TNF- α (Mathur *et al.*, 2002). In contrast, 14 days of supplementation with a juice rich in flavonoids (330 ml/day) enhanced *ex vivo* lymphocyte proliferation and lytic activity of NK cells associated with an increase in urinary excretion (Bub *et al.*, 2003). Soy milk consumption (16 weeks) was shown to decrease the percentage of blood monocytes (Huang *et al.*, 2005) and to increase the percentage of blood B cells (CD19⁺) without any effect on total T, CD4⁺ and CD8⁺ lymphocytes and NK cells (Ryan-Borchers *et al.*, 2006) in postmenopausal women.

A four-week supplementation with quercetin resulted in a significant increase in plasma quercetin concentration without affecting *ex vivo* LPS-induced TNF- α production (Boots *et al.*, 2008). Granulocyte respiratory burst activity, phagocytosis, NK cell activity and circulating counts of B, T or NK cells were not influenced by 12-week supplementation with quercetin at 500 or 1000 mg/day (Heinz *et al.*, 2010). Consumption of capsules containing fruit and vegetable concentrate for 77 days lead to an increase of $\gamma\delta$ -T cells, decreased DNA damage and *ex vivo* IFN- γ production from PMA-stimulated lymphocytes but had no effects on IL-4 and IL-6 (Nantz *et al.*, 2006).

15.7 Conclusion

The strong epidemiological evidence suggesting an inverse relationship between the intake of flavonoid-rich foods and chronic degenerative diseases has driven the scientific community to investigate the role of flavonoids in the modulation of immune response in humans, with a focus on its inflammatory component.

In vitro and *ex vivo* evidence suggests that flavonoids, at higher concentrations (between 10⁻⁵ M and 10⁻³ M), than those measured in the blood (between 10⁻⁹ M and 10⁻⁶ M), are able to modulate several aspects of the immune response. In particular they inhibit oxidative burst and the release of inflammatory cytokines, interfere with APC activity, reduce lymphocyte clonal expansion after challenge with different stimuli and preserve cell viability under oxidative stress conditions. The effect on Th1/Th2 cytokine balance suggests a decrease in Th1 response, but this aspect requires further and more convincing evidence. The immune-modulating action of flavonoids *in vitro* involves a number of well-described molecular mechanisms, including regulation of transcription factors such as NF κ B, AP-1 and Nrf2 and of membrane lipid rafts. However, the majority of these studies have been

conducted with non-physiological concentrations of flavonoids and not considering the wide array of flavonoid metabolites that appear in the bloodstream following ingestion of plant foods. Hence the modulatory activities of flavonoids, described in *in vitro* and *ex vivo* models, may not necessarily reflect effects that might be seen on *in vivo* immune response in humans.

To explain the gap between *in vitro* and *in vivo* observations, several considerations must be taken into account. First of all, the biological effects of flavonoids depend on their bioavailability, which appears to be low in humans. Secondly, once ingested, they are extensively metabolized into molecules with different chemical structure and activity compared to the ones originally present in the food. The large majority of *in vitro* experiments have been performed only with aglycones and not with the metabolites found in body fluids, increasing the chance of misinterpretation of the results. The few available studies involving biological metabolites indicate that, at concentrations close to post-ingestion circulating levels (10^{-6} M), these compounds are more active than the original ones in reducing oxidative burst and inflammatory cytokine secretion. Further complicating the picture, flavonoids may also regulate their own bioavailability and metabolism by modulating both activity and expression of multi-drug resistance (MDR) (Zhou *et al.*, 2004; Maher *et al.*, 2007; Bark and Choi, 2010; Klaassen and Reisman, 2010) and CYP450 (Ramadass *et al.*, 2003; Kang *et al.*, 2004; Chow *et al.*, 2006; Dong *et al.*, 2010) and, due to their antibacterial properties (Taguri *et al.*, 2004; Lee *et al.*, 2008; Anastasiadi *et al.*, 2009; Pastene *et al.*, 2009), affecting their microbial metabolism.

The experimental evidence from studies conducted in humans, despite some contrasting results, suggests a direct role for plant foods in modulating immune response *in vivo* as seen through a reduction of circulating plasma cytokine levels. However, the assumption that flavonoids might be responsible for the modulating effect of plant food is not fully justified on the basis of the current *in vivo* evidence. Most of the intervention studies lack assessment of flavonoid absorption or failed to associate the immunomodulatory effect following ingestion of plant foods with changes in circulating levels of flavonoids or their metabolites. Moreover, studies investigating the effect of flavonoids on markers of immune function have been mainly focused on flavonoid-rich foods and not on pure molecules. In this sense, only four (Zern *et al.*, 2005; Hsu *et al.*, 2007; Naruszewicz *et al.*, 2007; Egert *et al.*, 2008) out of the 14 studies utilizing flavonoids as supplements or food extracts, showed an effect on plasma cytokine levels. Moreover, when an increase of plasma flavonoid levels was described, no changes in markers of immune function were observed (Ryan-Borchers *et al.*, 2006; Nieman *et al.*, 2007a,b; Egert *et al.*, 2008; Heinz *et al.*, 2010) except for Egert *et al.* (2009), where quercetin absorption was associated with a reduction in circulating TNF- α .

Long-term intervention trials in humans with proper placebo and pure molecules involving techniques of metabolomics, gene arrays and a detailed analysis of immune response are needed to clarify if flavonoids represent ancillary ingredients or focal molecules involved in the immunomodulatory properties of plant foods.

15.8 Future trends

It has been recently speculated that the co-evolution of adaptive immune pathways, such as induced regulatory TGF- β -producing T cells (Treg) and T helper 17 cell lineages (Th17), and their specialized relationship to the gut, might have given primitive vertebrates a means to benefit from the diversification of their commensal microbiota (Weaver and Hatton, 2009). The gut is a peculiar environment, due to its constant contact with microbiota and food antigens (Peron *et al.*, 2009), that has important implication in immunonutrition particularly in oral tolerance through a cross-talk between bacteria, the epithelium and the immune system (Macdonald and Monteleone, 2005; Monteleone *et al.*, 2006). Due to low absorption of flavonoids, it is possible that the primary site of interaction between flavonoids and immune cells is the gut-associated lymphoid tissue (GALT). Future works should aim to understand if the gut environment might represent a feasible site where flavonoids may exert an action on the host immune system. In recent years, immunologists focused their attention on Th17 and Treg cells, due to their greater developmental plasticity than Th1 and Th2 cell lineages (Lee *et al.*, 2009b) and their role in regulating inflammation and autoimmunity (Fouser *et al.*, 2008; Li *et al.*, 2010). A few studies *in vitro* and in animal models have reported that flavonoids may modulate Treg (Mao *et al.*, 2003; Park *et al.*, 2010) and Th17 (Cho *et al.*, 2009; Kang *et al.*, 2009; Ye *et al.*, 2009; Park *et al.*, 2010) subsets. A potentially interesting area of research might investigate the role of flavonoids in modulating alternative partnerships involved in peripheral immunity.

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16

Immunomodulatory effects of mushroom β -glucans

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Abstract: Mushroom-derived dietary β -glucans have been shown to elicit diverse immunomodulatory effects in human and animal tissues, including the blood, gastrointestinal tract and spleen. In controlled human trials, β -glucan intake stimulated the immune system in the blood of healthy adults, dampened the allergic response to a respiratory inflammatory agent and improved survival in cancer patients. Additional randomized controlled trials are warranted to enable a more complete understanding of the immunomodulatory effects and specific applications of orally administered β -glucans.

Key words: mushroom, β -glucans, immunomodulation, diet, innate immunity.

16.1 Introduction

Mushrooms have immunomodulating properties, and for millennia they have been valued as tasteful foods and as medicinal substances. The knowledge and practice of the medicinal use of mushrooms originates from traditional eastern medicine, and in Japan, China, Korea and other Asian countries modern clinical practice still utilize mushroom-derived preparations. Since the 1980s scientific research has been performed on the medicinal properties of a growing number of mushroom species. Mushrooms are claimed to exhibit antiviral, antibacterial, cholesterol-lowering, blood pressure-lowering and hypoglycaemic effects. Mushroom products claiming to contribute to the treatment of infectious diseases, cardiovascular disorders and diabetes, and to the prevention of various other diseases are commer-

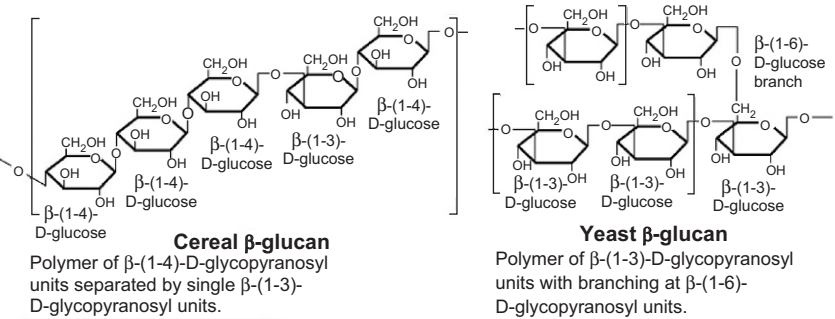
cially available. The most extensively studied application of medicinal mushrooms is their antitumour activity. Polysaccharides, mostly β -glucans, have been established to be one of the most potent antitumour-active compounds found in mushrooms. While most regular chemotherapeutic agents are based on direct cytotoxicity to cells, β -glucans mediate their antitumour activity by stimulating the immune system. Unfortunately, regular cancer therapy often has many serious side effects and can be unsuccessful. In addition, infectious diseases become increasingly difficult to treat, as pathogens are becoming resistant to many antibiotics. β -glucans may contribute to the solution of these problems by stimulating the immune system to enhance antitumour and anti-infective responses.

16.2 Introduction to β -glucans

Glucans are a class of glucose polymers and include starch ($(\alpha 1\text{--}4)$ -glucan) and cellulose ($(\beta 1\text{--}4)$ -glucan)). β -glucans are chains of D-glucose polysaccharides, linked by β -type glycosidic bonds. The six-sided D-glucose rings can be connected to one another, on a variety of positions on the D-glucose ring structure. Some β -glucan compounds are continual repeats of D-glucose attached at a specific position. Examples of these are $\beta(1\rightarrow3)$ -($1\rightarrow6$)-glucans, $\beta(1\rightarrow3)$ -($1\rightarrow4$)-glucans, and $\beta(1\rightarrow4)$ -glucans.

β -glucans occur widely and are structural cell wall components of yeasts, fungi, algae, lichens and bacteria. They are also found in a range of cereals (e.g. barley and oats where $\beta(1\text{--}3)$ -($1\text{--}4$)-glucans occur). There is considerable structural variation between β -glucans from different sources (Fig. 16.1). These differences can influence their physiological function and immunomodulating effects, and will also have an impact on the elimination half-life, volume of distribution and clearance. β -glucans can differ in type of linkage and branching of the glucose molecules resulting in variation in solubility, molecular mass, tertiary structure, charge and solution conformation (Brown and Gordon, 2003; Lehne *et al.*, 2006; Volman *et al.*, 2008; Chan *et al.*, 2009). This review focuses on β -glucans of fungal origin, which are $\beta(1\text{--}3)$ -linked glucose polymers, with varying patterns of branching. Lehne *et al.*, 2006; Volman *et al.*, 2008). The main types of branching in fungal β -glucans are $\beta(1\text{--}6)$ linkages.

Most β -glucans are considered to be non-digestible carbohydrates and are fermented to various degrees by the intestinal microbiota (Chan *et al.*, 2009). Specific information about the absorption of β -glucans is rare, but there may be some intestinal absorption. β -glucans exert immunomodulating effects in a variety of species, including humans, other mammals (ruminants, pigs), fish and poultry. Their effects are likely mediated via the innate immune system, e.g. via binding to a variety of receptors (for details see below), subsequent release of cytokines and stimulation of the humoral- and cell-mediated parts of the adaptive immune system (Williams, 1997; Brown and Gordon, 2003; Berner *et al.*, 2005; Volman *et al.*, 2008, 2010). To influence the immune system, β -glucans can be administered enterally (Volman *et al.*, 2008). They exert their effects via stimulation of the



β-glucan type	Structure	Description
Bacterial		Linear β1,3 glucan (i.e. Curdlan)
Fungal		Short β1,6 branched, β1,3 glucan (i.e. Schizophyllan)
Yeast		Long β1,6 branched, β1,3 glucan (i.e. WGP β-glucan, Betafectin™)
Cereal		Linear β1,3/β1,4-glucan (i.e. oat, barley, rye)

Fig. 16.1 The structure of different β-glucan sources (Volman *et al.*, 2008).

production of pro-inflammatory mediators, cytokines and chemokines, and upregulation of co-stimulatory molecules. At the cellular level, the binding and internalization of these carbohydrates proceeds in two phases: the first is rapid binding of the glucan ligand to the receptor, and the second is the slower uptake/internalization of the glucans (Williams, 1997). After internalization, the glucans eventually stimulate nuclear factor in κ-light chain producing B-cells (NFκB) activation, and this results in an increase in cytokine secretion (Brown and Gordon, 2003; Volman *et al.*, 2010) (Fig. 16.2). β-glucans are reported to be anti-infective, antitumourigenic and enhance the capacity of macrophage recognition and the capacity to clear apoptotic cells (Brown and Gordon, 2003).

16.2.1 Structure–function relationship of β-glucans

Much controversy surrounds the chemical and molecular principles of both the antitumour and immunomodulating activities of β-glucans. High molecular mass, the β(1→3)-glycosidic linkage, a triple helix and branches are considered favourable (Kulicke *et al.*, 1997). High molecular weight (1–2 × 10⁵ g/mol) fractions were active for immune stimulation, while fractions from the same source with molecular weights of 5–10 × 10³ g/mol showed no activity. The degree of branching (DB) is the number of single glucopyranosyl unit branches relative to the number of glucopyranosyl units in the linear chain. Biologically active glucans exist with DBs of 0.04–0.75; those with DB between 0.20 and 0.33 seem to be most active, for example schizophyllan and grifolan. It should be noted that lentinan is

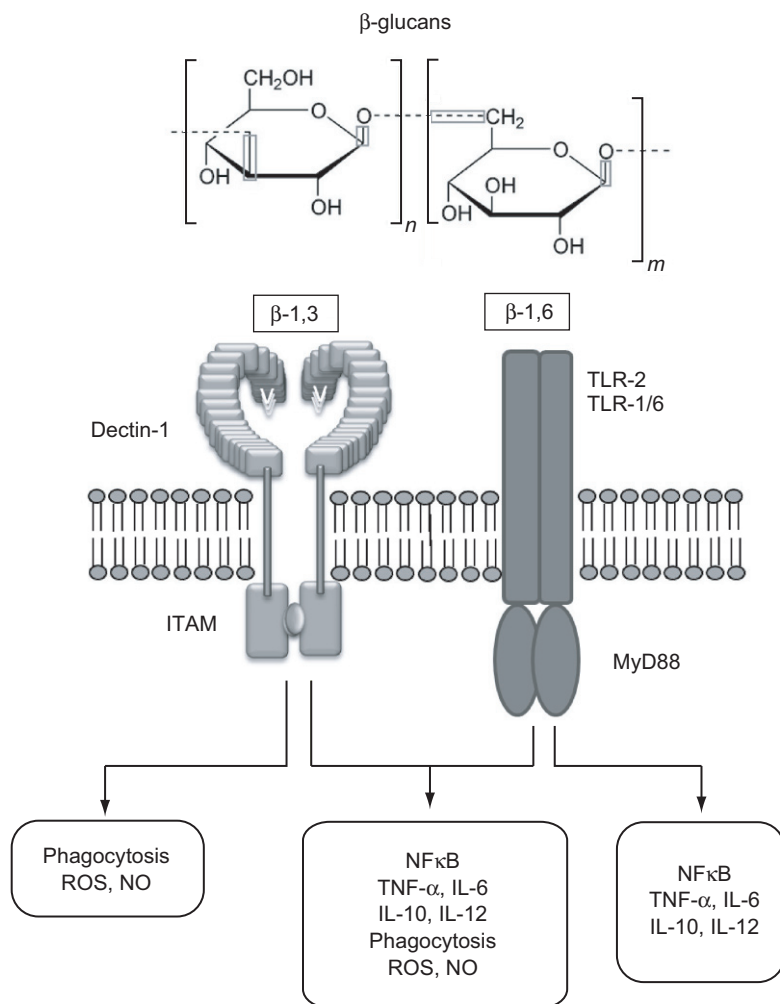


Fig. 16.2 Conceptual model for the interaction between β -glucans and receptors of the innate immune system (macrophages). Interaction between Dectin-1 and toll-like receptors (TLRs) results in synergistic effects. ROS, reactive oxygen species.

considered to have a DB of 0.33 in contrast with the generally accepted DB of 0.4 (Bohn and BeMiller, 1995). β -glucans exist in three conformations: single helix, triple helix and random coil. Only molecules of higher molecular weight ($>9 \times 10^4$ g/mol) appear to form triple helical structures. Lentinan and schizophyllan have a triple helix structure as their native conformation. Stimulation of T cell-mediated responses was found to be decreased by denaturation of lentinan. Thus, triple helix structure appears to be necessary for the most effective immunological stimulation (Falch *et al.*, 2000).

16.3 Immunological activity of mushroom β -glucans

The immune activities of mushroom-derived polysaccharides are well documented in rats and humans. These appear to promote the activity of antigen non-specific natural killer (NK) cells that are able to rapidly and effectively kill cancerous cells; enhance the production of interferons that effectively prevent virus reproduction; increase the activities of complement C3 that enhances resistance; increase the number and activities of phagocytes (neutrophilic granulocytes) that release H_2O_2 which is toxic to cancerous cells (Wargovich *et al.*, 2010). As immunomodulators, polysaccharides affect the growth of immune organs (bursa, thymus, and spleen), activities of immune cells (granulocytes, monocytes, macrophages and NK cells), and functions of both cellular immunity and humoral immunity (T cells and B cells) (Lien, 1990; Xue and Meng, 1996; Guo *et al.*, 2003; Li *et al.*, 2010). β -glucans increased spleen and thymus index, rate of T-lymphocyte transformation and proliferation as well as production of interleukin (IL)-2, while decreasing the inhibiting effects of macrophages and suppressor/regulatory T cells on T-lymphocytes in spleen, thymus and lymph nodes. They enhanced the humoral immune response by increasing spleen antibody production and serum antibody titres.

16.3.1 Mushroom β -glucans and receptor activation

Pattern-recognition receptors (PRRs), located on the surface of innate immune cells, recognize conserved molecular structures of invading pathogens called pathogen-associated molecular patterns (PAMPs) (Janeway, 1992). Mushroom β -glucans are themselves PAMPs in which their specific interaction to PRRs induces cell-mediated and humoral immune responses (Lull *et al.*, 2005; Akramienė *et al.*, 2007; Borchers *et al.*, 2008). Toll-like receptors (TLRs) are localized in the cell membrane and/or endosomal membrane components to recognize extracellular and endocytosed PAMPs; 13 TLRs have been reported in mice and humans. For TLRs, it has been shown that ligand sensing and specificity is achieved through the arrangement and sequence variation in conserved leucine-rich repeat (LRR) domains. A variety of PRRs are responsible for β -glucan recognition; for instance Dectin-1, CR3, lactosylceramide and scavenger receptors (SRs) (Brown and Gordon, 2001; Ujita *et al.*, 2009). These receptors are positioned on specific cell surface areas of monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils and NK cells, and on some non-immune cells such as enterocytes, alveolar cells and fibroblasts (Thompson *et al.*, 2010).

Complement receptor 3 (CR3) is an integrin dimer consisting of CD11b non-covalently associated with CD18 (Thornton *et al.*, 1996; Thompson *et al.*, 2010). It is expressed on neutrophils, NK cells, DC, and T and B lymphocyte subsets, and is less strongly expressed on macrophages (Ross, 2000). A fully activated neutrophil may express on its membrane 200 000 or more CR3 molecules. β -glucan binding to CR3 activates the iC3b pathway causing tumour cytotoxicity. The binding of β -glucans to neutrophils is dependent on CR3, unlike macrophages whose β -glucan-binding is CR3-independent (Li *et al.*, 2006; Chan *et al.*, 2009).

Lactosylceramide (LacCer; CDw17) is found on the plasma membrane of several cell types. It has been suggested that the interaction of β -glucan with this receptor can induce macrophage inflammatory protein (MIP)-2 and the activation of NF κ B (Akramienė *et al.*, 2007).

SRs are a family of cell surface glycoproteins found on myeloid and endothelial cells (Rice *et al.*, 2005; Chen and Seviour, 2007). CD36 and SCAF1 from this family have been found to bind β -glucans. It has been reported that binding of β -glucan to some selected SRs could help endotoxin clearance (Vereschagin *et al.*, 1998).

16.3.2 Dectin-1 and innate immunity

Dectin-1 was the first identified β -glucan receptor with a profound role in innate recognition of highly conserved β (1-3) and/or β (1-6) glucans (Brown *et al.*, 2002; Taylor *et al.*, 2002). Dectin-1 is a type II membrane receptor that belongs to the C-type lectin family of receptors, containing an extracellular C-terminal C-type lectin domain, a short stalk region, a single transmembrane domain and a short 40 amino acid intracellular amino terminal tail (Willment *et al.*, 2005). The receptor is expressed primarily by cells of myeloid origin, including macrophages, DCs and neutrophils in mice. In addition, Dectin-1 has been detected by flow cytometry on a subset of B and T lymphocytes although its function on these cells is undetermined (Adams *et al.*, 2008). Dectin-1 signals contribute to a variety of macrophage, DC and neutrophil responses, including phagocytosis, oxidative burst, neutrophil degranulation, fungal killing and the production of inflammatory lipid mediators, cytokines and chemokines that recruit and coordinate the activation of other immune cells. In general, Dectin-1 does not bind oligosaccharides comprising less than seven glucose units (Adams *et al.*, 2008). Dectin-1 can cooperate with other receptors, like TLR2 and TLR4 for high affinity recognition (Underhill *et al.*, 2005; Brown, 2006). Dectin-1 binds the carbohydrate polymers with affinities ranging from very low (3×10^{-3} M) to very high (2×10^{-12} M). The wide range of affinities appears to be due to the differing sizes and numbers of branches in β -glucans from various sources (Goodridge *et al.*, 2009).

The crystal structure of the lectin domain suggested that while individual Dectin-1 molecules can bind β -glucans, cooperative binding as dimers may be more efficient (Banchereau *et al.*, 2000). The ability of Dectin-1 to directly activate NF κ B and drive cytokine induction depends on the specific cell type and micro-environment. Dectin-1 can collaborate with TLRs, including TLR2, TLR4, TLR5, TLR7 and TLR9, to amplify TLR-mediated NF κ B activation and cytokine induction, even in cells where Dectin-1 signals cannot activate NF κ B directly. Furthermore, Dectin-1 directly induced tumour necrosis factor- α (TNF- α) production by DCs, resident peritoneal cells and alveolar macrophages, but not by other macrophages. Thus certain cell populations are intrinsically wired for cytokine induction upon Dectin-1 ligation, while others are not. Induction of cytokine production by β -glucan particles is also regulated by mitogen-activated protein (MAP) kinases. Dectin-1 signals activate NFAT (nuclear factor of activated

T-cells) transcription factors in activated macrophages and DCs, and NFAT signalling regulates cyclooxygenase-2 (COX-2) and prostaglandin production by macrophages, and the induction of IL-2, IL-10, IL-12 p70 and IL-23 by DCs (Goodridge *et al.*, 2009).

Stimulation of Dectin-1 on DCs preferentially directs the subsequent polarization of Th17 cells producing large amounts of IL-23. However, the role of glucan-stimulated Th17 polarization in coordinating effective host defence against fungal infections is more difficult to establish. The control of fungal infections has classically been attributed to an effective innate antimicrobial immune response that is enhanced by the cytokines produced by Th1 cells. Recent studies have explored the contribution of Th17 and T-regulatory cells (Tregs) in controlling fungal infections, albeit with varying results. Additional regulation of the immune response to fungal pathogens is provided by Tregs, which are thought to help prevent excessive pathology due to the immune response as well as contribute to the development of persistent immune memory.

16.3.3 Cooperation of Toll-like receptors and Dectin-1

β -glucans were found to interact with TLRs and both TLR2 and TLR6 are required for activation of NF κ B in macrophages and DCs, leading to the production of the pro-inflammatory cytokines TNF- α and IL-12 (Fig. 16.2). The adaptor protein MyD88 mediates the intracellular signalling to NF κ B. The binding of β -glucans to Dectin-1 alone does not stimulate the production of TNF- α and IL-12. However, Dectin-1 enhances the production of these pro-inflammatory cytokines when TLR2 is stimulated (Gantner *et al.*, 2003). This suggests that the strong pro-inflammatory activities reported for β -glucans in many studies, might be partly caused by unidentified TLR2 triggering contaminants in impure extracts. This hypothesis is supported by the results of a study performed by Kataoka *et al.* (2002) who found that branched (1 $\rightarrow$ 3) β -glucans, like lentinan and schizophyllan, could not stimulate NF κ B activity in macrophages.

The activation of Dectin-1 alone by β -glucans is sufficient to stimulate the respiratory burst and phagocytosis. Mouse B cell hybridoma cells (which normally cannot bind zymosan) were transfected with Dectin-1 and were then able to bind β -glucans and produced IL-2 and IL-10. This shows that cytokine production can also occur independent of TLR signalling. A kinase called Syk was recruited upon β -glucan binding to Dectin-1. A single phosphorylation of the membrane-proximal tyrosine in the ITAM-motif was sufficient to recruit Syk and to couple to downstream IL-2 and IL-10 responses, but not for IL-12 synthesis which could be signalled via the TLR2 pathway (Rogers *et al.*, 2005).

16.3.4 Mushroom β -glucans and cellular activation, cytokine and antibody responses

The immunomodulating properties of β -glucans, in innate and adaptive immunity, and their anti-inflammatory properties have been widely investigated. β -glucans

induce NF κ B gene expression via Dectin-1 in association with TLR-2 and -4 in a MyD88-dependent signalling cascade (Thompson *et al.*, 2010). Up-regulation of the NF κ B gene is considered significant as an important regulator of cellular activation, cell cycle progression, cell survival, cell adhesion, invasion and inflammatory responses (Ravi and Bedi, 2004). Mushroom β -glucans increased phagocytosis activity, nitric oxide production and catalase activity of macrophages, and delayed-type hypersensitivity and reduced tumour formation in a mouse model (Zheng *et al.*, 2005). The production of inflammatory cytokines and mediators induced by mushroom β -glucan improves the ability to fight against viral, bacterial and fungal challenges to innate immunity.

DCs are key connections between innate and adaptive immunity. They are antigen presenting cells which have the special ability to prime naïve CD4⁺ T cells to polarize to helper T cell responses (Th1 or Th2) resulting in cell-mediated or humoral immune responses (Banchereau *et al.*, 2000; Borchers *et al.*, 2008). It has been reported that skewing of Th1 vs Th2 can be regulated by β -glucans isolated from different mushrooms (Murata *et al.*, 2002; Kupfahl *et al.*, 2006).

Not only T lymphocytes but also B lymphocytes can be regulated by mushroom β -glucans. β -glucan from *Ganoderma lucidum* was found to induce B lymphocyte differentiation into IgM-secreting plasma cells (Lin *et al.*, 2006). Dietary supplementation in animal models with crude water-soluble polysaccharide from *Lentinus edodes*, *Tremella fuciformis* and *Agaricus blazei* increased specific IgA, IgM and IgG concentrations (Guo *et al.*, 2004; Chan *et al.*, 2007). However, B-lymphocyte function was suppressed by a polysaccharide fraction isolated from *Ganoderma lucidum* and *Grifola frondosa* in a mouse model (Bao *et al.*, 2001; Inoue *et al.*, 2002).

16.4 Immunomodulation by mushroom β -glucans as dietary components

Mushrooms contain polysaccharides, proteins and various small molecular weight compounds such as vitamins and minerals (Wasser, 2003). The main extraction procedure for isolation of β -glucans commonly begins with hot water extraction followed by alcohol precipitation. However, differences such as extraction time, temperature and post-extraction techniques can affect the purity and activity of the β -glucans isolated (Yadomae, 2000; Brown and Gordon, 2003; Hong *et al.*, 2003).

16.4.1 Oral delivery and gastrointestinal absorption

Rice *et al.* (2005) examined the absorption, pharmacokinetics and biological effects of the water-soluble β -glucans glucan phosphate, laminarin and scleroglucan that were administered by oral gavage to rodents. Laminarin (molecular weight 7.7×10^3 ; DB: 1/10; single helix) and scleroglucan (molecular weight 1.02×10^6 ; DB 1/3; triple helix) both have a backbone of (1 $\rightarrow$ 3)- β -D-glucopyranosyl units and β (1 $\rightarrow$ 6)-linked side chains, and scleroglucan is comparable to the mushroom

β -glucans. Oral administration produced measurable plasma levels of the β -glucans. Laminarin showed two peak plasma levels: one at 3 hours and one at 12 hours, while scleroglucan plasma levels peaked twice as well: at 15 minutes and at three hours. Scleroglucan, the largest β -glucan, was absorbed most rapidly. The maximum plasma level was 115 ng/ml for laminarin and 355 ng/ml for scleroglucan when rats were given an oral dose of 1 mg/kg. It is noteworthy that most *in vitro* studies are performed with higher concentrations of β -glucans (approximately 5–500 μ g/ml), so it is questionable if the low plasma levels after oral administration are sufficient for immunostimulation. The bioavailability of laminarin and scleroglucan was 4.9 and 4.0%, respectively. A water-insoluble, particulate glucan preparation was not detected in plasma as this is most likely phagocytosed and transported by macrophages.

Fluorescently labelled β -glucans were used to demonstrate that gut-associated lymphoid tissue (GALT) cells, isolated from Peyer's patches, can bind β -glucans. Macrophages showed an increase in Dectin-1 expression and DCs increased their TLR2 expression. Only a subpopulation (10%) of intestinal epithelial cells incorporated β -glucan; such cells did not express Dectin-1 receptor, and uptake of β -glucans probably occurred through microfold (M) cells in the intestine (Hashimoto *et al.*, 1991). A different explanation might be a prebiotic effect of β -glucans on the gut flora as was shown for oat glucans (Charalampopoulos *et al.*, 2002). Taken together, these experiments show that orally administered β -glucans interact with a variety of gastrointestinal cells, enter the systemic circulation and persist in the plasma up to 24 hours.

16.4.2 β -Glucan path from the diet to gut immune cells

Due to the absence of the enzyme β (1-3) glucanase in vertebrates, orally administered β -glucan can resist the oral cavity and stomach, and thus transit in intact form to the small intestine to come in contact with enterocytes, located as the frontline of intestinal immune system (Nono *et al.*, 1991; Thompson *et al.*, 2010). The layer of enterocytes is occasionally interposed by specialized cells called M cells which take up and transport particulate antigens from the gut lumen across the epithelial barrier to the underlying loosely organized immune cells, like macrophages, T cells and B cells (Brandtzaeg *et al.*, 2008). DCs are a cell type which has the special ability to penetrate their dendrites between the epithelial cells to sense harmful particles directly from the gut lumen and present peptide fragments of the luminal antigens to the immune cells located underneath the epithelium (Soloff and Barratt-Boyes, 2010). DCs are the most important professional antigen presenting cells and express up to 100 times more major histocompatibility complex (MHC) and are more effective at differentiating naïve T cells than other antigen presenting cells. This intestinal immune system is referred to as Peyer's patches and 100–600 of these can be found in the human gut. All of the typical immune cells are present in the Peyer's patches, such as macrophages, DCs, B- and T-lymphocytes (Brandtzaeg *et al.*, 2008). It has been reported that orally administered fluorescently labelled β -glucans were present in immune cells isolated from Peyer's patches

after 24 hours of feeding in a mouse model. However, differences in solubility, size and structure of β -glucans affect their recognition by β -glucan receptors on these cells, bioavailability and concentration in the systemic circulation (Rice *et al.*, 2005).

16.4.3 Distribution, metabolism and excretion

Suda *et al.* (1996) studied the distribution and metabolism of i.p. administered, labelled SSG (a β -glucan from *Sclerotinia sclerotiorum*) in mice. Following administration, the concentration of SSG was first high in peritoneal exudate and blood, but these concentrations lowered sharply over 48 hours while concentrations increased mainly in liver and spleen, and a slight increase was found in the kidney, intestine and faeces. After one month, about 30% of administered SSG was present in the liver and about 10% in the spleen. Because of the absence of (1 $\rightarrow$ 3)- β -D-glucanase in mammals, these glucans are thought to be metabolized by oxidative degradation by macrophages. However, the majority of SSG in liver and spleen was recovered from the non-cellular fraction and not from macrophages. These results suggest that β -glucans are not easily taken up by macrophages. The elimination of β -glucans from the blood is quite slow. After a single i.v. dose (1 mg/kg) administered to rats, the elimination half-life was 2.6 hours for laminarin and 3.1 hours for scleroglucan (Rice *et al.*, 2004). The i.p. administration of multiple doses of β -glucans (0.25 mg/week) to mice led to saturation of the liver, spleen and circulation with glucan (Miura *et al.*, 1995). Not much is known about the clearance of β -glucans from the whole body. Lower molecular weight β -glucans are possibly cleared by glomerular filtration in the kidney. High molecular weight glucans (such as lentinan) are retained mainly in the liver and degraded by liver macrophages, the Kupffer cells, which may take several weeks.

16.4.4 *In vitro* studies

The immunomodulating properties of mushroom β -glucans have been demonstrated in many *in vitro* studies in recent years. A variety of immune cells (either cell lines or primary cells), such as monocytes, macrophages, DCs, neutrophils and T-lymphocytes, have been used in such studies (Kupfahl *et al.*, 2006; Borchers *et al.*, 2008). Peripheral blood mononuclear cells (PBMCs), bone marrow-derived macrophages and DCs, alveolar macrophages, spleen cells and epithelial cell lines like the Caco-2 have all also been used (Chaung *et al.*, 2009; Lin *et al.*, 2010; Volman *et al.*, 2010). Different forms of mushroom β -glucans, for instance, crude mushroom extract (Guo *et al.*, 2004), pure isolated native β -glucans (Lin *et al.*, 2005; Kupfahl *et al.*, 2006; Lin *et al.*, 2010), modified isolated β -glucans (Rice *et al.*, 2005) and fractionated β -glucans (Inoue *et al.*, 2002), have been examined for their immunomodulating properties.

Enhanced innate immune responses are accomplished by an increased pro-inflammatory cytokine and chemokine production after stimulation by mushroom

β -glucans (Zheng *et al.*, 2005; Borchers *et al.*, 2008; Thompson *et al.*, 2010). Simultaneous stimulation of lipopolysaccharides (LPS) and mushroom β -glucans increased IL-6 and TNF- α production in a murine macrophage cell line (Kim *et al.*, 2009). Anti-inflammatory activities (Ellertsen *et al.*, 2006), the ability to activate naïve T lymphocytes (Murata *et al.*, 2002) and even anticancer properties (Angeli *et al.*, 2009) of mushroom β -glucans have been reported in *in vitro* studies. Interactions of β -glucans and their PRR, including the downstream regulatory pathways, have been mostly studied in *in vitro* systems. Summarizing, it can be concluded from *in vitro* studies that mushroom β -glucans enhance the immune responses of leukocytes and epithelial cells, mainly by modulating cytokine/chemokine production.

16.4.5 Animal and human studies

In addition to *in vitro* studies, numerous animal studies have been conducted to evaluate the immunomodulating effects of mushroom β -glucans. Most studies have been carried out with PBMCs isolated from animals that were treated with β -glucan supplied via oral and systemic administration. Subsequently, these isolated cells were challenged with LPS or pathogen. In general, these *ex vivo* experiments showed similar finding as *in vitro* assays (Kasai, 2004; Kupfahl *et al.*, 2006). *In vivo* effects of mushroom β -glucans have been reported for the response of pathogen-infected animals. These studies showed improved microbial clearance and reduced mortality of lethally infected animals given β -glucans. For instance, there were indications that the bacterial load in the spleen and liver was significantly reduced during bacterial infection of lentinan pre-treated mice (Kupfahl *et al.*, 2006). Pro-inflammatory cytokines and NO production were increased in lentinan-fed mice (Zheng *et al.*, 2005). Controlled human and animal studies have shown anti-inflammatory and anti-allergic effects of some polysaccharide products. Orally applied finely dispersed lentinan significantly diminished symptoms of cedar pollen-induced rhinitis, and also perennial allergy (Yamada *et al.*, 2007). However, limited human studies have been carried out on mushroom glucans. In most studies, crude mushroom extracts were used. The powdered fruiting body of shiitake (*Lentinus edodes*), the mushroom from which lentinan is extracted, has antitumour activity when administered orally as 20% of the diet of mice. Shiitake feeding was effective in inhibiting carcinoma growth and improving the spreading rate and phagocytosis of macrophages, the mitogenic response of lymphocytes to concanavalin A, and the cytotoxic activity of NK cells (Borchers *et al.*, 2004). It would be useful to search for the lowest dose for which immunomodulatory effects are still observed.

16.4.6 Mushroom β -glucans and preventive health effects

Mushroom β -glucans have also been demonstrated to show antioxidant, anti-mutagenic and anticarcinogenic properties (Kassie *et al.*, 2002; Guerra Dore *et al.*, 2007). The protective effect was shown by the reduction of DNA damage in

PBMCs pretreated with mushroom β -glucans before exposure to mutagens. As an explanation, the binding ability to mutagens or the capturing ability for free radicals of mushroom β -glucan was proposed (Angeli *et al.*, 2006). Also, mushroom β -glucan showed inhibition of cancer cell proliferation and inactivated enzymes involved in formation of reactive metabolites (Hashimoto *et al.*, 2002; Zhang *et al.*, 2006). It has been demonstrated that pre-incubation of immune cells with mushroom β -glucans prior to bacterial or protozoan infection resulted in a significant production of pro-inflammatory cytokines (Kim *et al.*, 2003). Dietary polysaccharides appear to be at least partially degraded by bacterial enzymes in the human digestive tract and are known to impact gut microbial ecology which can impact host nutrition, immune modulation, resistance to pathogens, intestinal epithelial development and activity, and energy metabolism (Ramberg *et al.*, 2010).

16.5 Safety of dietary β -glucans

Data on acute and chronic toxicity to humans and adverse reactions to β -glucans are very limited. In mice, there were no significant treatment-related effects on pregnancy outcomes and litter characteristics. To test the chronic toxicity of an overdose of β -glucan, rhesus monkeys were followed for six months. Two significant effects comprised a general foam cell reaction and vasculitis (inflammation of blood vessels). The foam cells are macrophages, which are filled with phagocytosed undigested polysaccharide and/or lipid. The varying degrees of vasculitis and associated reactions accounted for the clinical signs. Side effects in humans were studied for β -glucan that was administered i.v. to 50 cancer patients, and abnormality in liver function test, mild oppression on the chest and petechiae were reported (Taguchi, 1980; 1987). From the fact that β -glucan caused petechiae at a dosage of 50 mg/person/day, the researchers recommended a dose of 0.5–5 mg/person/day i.v. Caution should be taken when β -glucans are combined with other drugs as the combination of a β -glucan (schizophyllan, SSG or grifolan) and the anti-inflammatory drug indomethacin induced lethal toxicity in mice (Yoshioka *et al.*, 1998). All of the polysaccharides which caused this effect were branched β -glucans with strong immunomodulating activities.

Toxicological data on β -glucans are very limited; at a low dose of 0.5 mg/kg/day no adverse effects were observed. Taking into account safety factors for inter- and intraspecies differences in sensitivity, a much lower dose would be recommended for humans. In therapeutic use against cancer, a higher dose may be acceptable as side effects of β -glucans may be less severe than those of other chemotherapeutic agents as these are not directly cytotoxic. In clinical studies a dosage of about 2 mg/person i.v. or i.p. is often used, as a single or weekly dose (Oka *et al.*, 1992; Hamano *et al.*, 1999). β -glucan is taken up by eating shiitake mushrooms. Assume that 2 mg lentinan/week would be a dosage that has immunostimulatory effects in humans, that shiitake mushrooms are consumed

after a week of storage at 5 °C, and a bioavailability of 4%. The fruiting body would contain 10–300 mg β -glucan after storage. So a weekly consumption of approximately 25–450 grams of shiitake mushroom would deliver 2 mg of absorbed β -glucan to the body (Minato *et al.*, 1999; Yap and Ng, 2001).

Most polysaccharide products appear to be safe, based on NOAEL (no observed adverse effect level), acute and chronic toxicity testing in rodents. As would be expected, powders, extracts and products that have not been fully characterized pose the most concerns. The impact of polysaccharide intake on the absorption of nutrients and medications is not known. Product contamination may often explain the occasionally observed adverse side effects. The literature is highly heterogeneous and is not sufficient to support broad structure/function generalizations. For the limited number of studies that investigated well-characterized, isolated products (primarily β -glucan products), effects can be unequivocally attributed to polysaccharides. Such associations are certainly more tenuous when considering product powders or products obtained by extraction methods designed to isolate polysaccharides, but without complete compositional analyses.

Additional tests of well-characterized products are needed to more completely understand the immunomodulatory effects and specific applications of oral polysaccharides. Such studies will need to better investigate the optimal timing and duration of polysaccharide ingestion – should they be consumed continuously, before, at the time of, or after exposure to a pathogen or environmental insult? Only a few studies have actually investigated the impact of timing of polysaccharide intake to achieve optimal benefits. Daily feeding with some polysaccharides appears to result in tolerance (and diminished benefits); this has been demonstrated for some mushroom β -glucans. For those polysaccharides whose immunologic effects are dependent on their prebiotic activities, regular feeding would presumably be necessary.

16.6 Conclusion and future trends

β -glucans modulate the immune system by binding to specific receptors including Dectin-1, CR3, TLR2, TLR6, scavenger receptor and lactosylceramide. For most receptors the mechanisms and responses are still not entirely clear. Therefore more research is necessary on the mechanisms behind β -glucan receptor-binding and the subsequent cascade of effects. Binding of β -glucan to its receptor will result in the activation of immune cells and in the activation of NF κ B, or in the activation of cytoplasmic p38MAPK, which will impact on cytokine production. The activation of NF κ B leads to a cascade of reactions causing transcription of immune genes. NF κ B plays a crucial role in inflammatory gene transcription. After activation of NF κ B, the immune system is better able to resist attacks by pathogens. The activation of p38MAPK results in the activation of enzymes and transcription factors, which leads to improved immune response. It is necessary to do more

research on these two signal transduction molecules, because the complete mechanisms and the function of each molecule remain unknown.

The activation of immune cells and the unblocking of NF κ B will result in increased release of cytokines. β -glucans enhance the systemic level of pro-inflammatory cytokines and cytokines involved in cell-mediated immunity. Due to a higher cytokine level and higher mRNA expression, levels of NO are also enhanced, improving host defence mechanisms. Due to β -glucan exposure, levels of cytokines that regulate and control inflammation and infection reactions will be increased. Modest administration of β -glucan is recommended because of the beneficial effects of β -glucan on the immune system.

Most studies on β -glucans were carried out on cells and animals. There are limited clinical trials in humans from which we can conclude that β -glucans are safe and with beneficial effects. β -glucans tended to prevent infections and reduce mortality after surgery, and faster recovery of the patients was observed. The patients subsequently needed fewer antibiotics. In addition β -glucans appear to exert beneficial effects against allergies and cancer.

β -glucans show potential to attenuate a variety of immune diseases. Through the NF κ B signalling cascade, it can be a promising therapeutic target for inflammatory and autoimmune diseases. β -glucans can be administered parentally or enterally. There is no difference in protection against pathogen infections after parental or enteral administration. This implies that fortification of food with β -glucans may be used to modulate immune function, and might improve resistance against invading pathogens in humans. The effect after enteral administration of β -glucan seems independent of the source of β -glucan used. Therefore, enriching the diet with β -glucans may be an important method of enhancing protection against pathogens. In animals, this application is already common. This suggests that β -glucans have a role in functional foods and as dietary supplements.

A better understanding of the mechanisms of β -glucan recognition will guide attempts to improve host defence against fungal pathogens and to utilize soluble and particulate β -glucans to modulate innate and adaptive immune responses. Although the main receptors involved in β -glucan recognition may already be identified, it is likely that others remain to be described. Optimal exploitation of β -glucans in anticancer treatments and vaccines, and in priming host defences will require a mechanistic understanding of how each receptor works. We will need a complete model for how each receptor signals to understand how β -glucans are similar to and different from other microbe-derived compounds being evaluated.

In conclusion, β -glucans can be used to improve immune responses. Possibilities for application include functional foods, food supplements or via parental administration, as a medicine. β -glucans may be a suitable therapeutic agent for the future to mitigate several immune-related diseases, such as allergies, cancer and infections, and they can improve resistance against invading pathogens in humans. Currently, more research is needed to determine the complete mechanism through which β -glucans are working.

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17

Modulation of inflammatory and immune responses by short-chain fatty acids

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Abstract: Short-chain fatty acids (SCFAs) are the main metabolic products of anaerobic bacterial fermentation in the intestine. In addition to their important role as fuel for intestinal epithelial cells, SCFAs modulate different processes in the gastrointestinal (GI) tract and in other tissues such as adipose and immune tissues. In this chapter, we first present a brief description of basic aspects of SCFA physiology, such as production, absorption and metabolism. The main mechanisms by which SCFAs act on cells and the effects of these fatty acids in isolated leukocytes are then discussed. We also highlight the results obtained by studies performed *in vivo* using SCFAs in the treatment of inflammatory pathologies. A brief discussion about the perspectives in this research field is presented at the end of the chapter.

Key words: short-chain fatty acids, inflammation, immune response, GPR43 and histone deacetylase.

17.1 Introduction: physiology of short-chain fatty acids (SCFAs)

Human mucosal surfaces are colonized by an astonishing number of micro-organisms (it is estimated that 90% of the cells in the human body are microbial) of different kingdoms (i.e. fungi and bacteria) and genera (i.e. *Bifidobacterium*, *Eubacterium*, *Fusobacterium*, *Escherichia* and *Candida*). These non-human cells are collectively referred as microbiota or microflora. A complex and dynamic interaction among the microbes themselves and between them and their host regulates both microbial composition and host physiology and pathology as discussed below (Hooper *et al.*, 2002; Guarner and Malagelada, 2003).

The microbiota of the gastrointestinal (GI) tract exert protective (i.e. natural defence barrier and production of antimicrobial factors), structural (i.e. immune system development and induction of IgA) and metabolic (i.e. fermentation of non-digestible dietary residues, synthesis of vitamins and promotion of mineral absorption) functions (O'Hara and Shanahan, 2006). The composition of the gut microbiota depends on both the host genotype and environmental factors; it fluctuates under some circumstances such as diarrhoea and antibiotic therapy but, once, established usually remains fairly constant (Guarner and Malagelada, 2003; O'Hara and Shanahan, 2006).

A major metabolic contribution of the gut microbiota is through fermentation of non-digestible dietary residues and endogenous epithelial-derived mucus, a process by which the micro-organisms of the GI tract recover a great amount of energy for the host. Recent studies indicate that the gut microbiota, acting through different mechanisms such as harvesting energy from the diet and regulating the synthesis of gut peptides and fat storage, modulates energy homeostasis in the host and can be involved in the development of metabolic disorders such as obesity and diabetes (Cani and Delzenne, 2009; Gao *et al.*, 2009; Schwartz *et al.*, 2010).

Short-chain fatty acids (SCFAs) are the main metabolic products of anaerobic bacterial fermentation in the intestine (see Plate I in colour section between pages 516 and 517). The predominant SCFAs are acetate (C_2), propionate (C_3) and butyrate (C_4). These fatty acids result mainly from carbohydrate fermentation, a process in which monosaccharides, generated from carbohydrate polymers by microbial enzymatic activity, are initially converted to pyruvate and then to SCFAs. Protein fermentation in the GI tract also contributes to SCFA synthesis in the colon (Rasmussen *et al.*, 1988). The concentration of SCFAs in humans is approximately 13 mM in the terminal ileum and ranges from ~130 mM in the caecum to ~80 mM in the descending colon (Cummings *et al.*, 1987) with an approximate relative ratio of 70:20:10 (acetate, propionate and butyrate, respectively) (Cook and Sellin, 1998). However, these concentrations are affected by various factors such as diet and composition of intestinal microbiota (Hooper *et al.*, 2002).

SCFAs produced in the intestine are readily absorbed from the lumen by at least two mechanisms: (i) simple diffusion of the protonated form and (ii) absorption of the ionized form by a mechanism involving the co-transport of Na^+ or K^+ (Topping and Clifton, 2001). Modifications in the amount of SCFAs produced in the intestine affects fluid and electrolyte absorption. SCFA absorption stimulates Na^+ -dependent fluid absorption through apical membrane Na^+-H^+ , $SCFA-HCO_3^-$ and Cl^- -SCFA exchanges (Binder, 2010).

SCFAs are relatively low energy contributors in humans (it is estimated that 5–10% of human basal energy requirements are provided by SCFAs) (McNeil, 1984) compared to other species, especially ruminants, where they can provide up to 80% of energy requirements. Colonocytes are especially dependent on SCFAs as source of energy (60–70% of energy for these cells is supplied by oxidation of SCFAs). The order of substrate utilization by colonocytes is butyrate > acetate and propionate > glucose and glutamine (Fleming *et al.*, 1991; Clausen and Mortensen, 1995).

After absorption, some acetate and propionate, which are less metabolized by colonocytes, but also butyrate, cross the epithelial colonic cells, reach the blood and are transported to the liver (Plate I).

Propionate and butyrate are almost totally taken up by the liver from the bloodstream, while the liver absorbs almost half of the plasma acetate (Rémésy *et al.*, 2004). In the liver, SCFAs are used as energetic substrates and as precursors of other molecules such as glucose (propionate is a substrate for gluconeogenesis), fatty acids and ketone bodies. Despite some controversy in the literature, serum concentrations of acetate, propionate and butyrate have been shown to be in the range 19–227 μM , 1–14 μM and 1–16 μM , respectively (Cummings *et al.*, 1987; Wolever *et al.*, 1997; Zhao *et al.*, 2007). SCFAs not used by the liver are taken up by other tissues such as skeletal muscle, heart and brain, where they are used mainly as energy sources. Blood acetate is not derived uniquely from the colon, since several tissues, especially the liver, can also produce and release it (Yamashita *et al.*, 2001).

In addition to their important role as fuels for intestinal epithelial cells, SCFAs modulate different processes in the GI tract such as electrolyte and water absorption (Binder and Mehta, 1989) and colonic motility (Cherbut *et al.*, 1998). These fatty acids are also able to modulate adipogenesis (Hong *et al.*, 2005), cellular proliferation and differentiation (Millard *et al.*, 2002; Hossain *et al.*, 2006; Wang *et al.*, 2008) and immune function. Most of these effects are attributed, at least in part, to the action of these fatty acids on the GPR41 and GPR43 receptors and on the activity of histone deacetylase (HDAC) as discussed in the following section.

17.2 Short-chain fatty acids mechanisms of action

There are at least two well-established mechanisms by which SCFAs affect cell function: (i) activation of G protein-coupled receptors (GPR41 and GPR43) and (ii) inhibition of HDAC activity. A brief description of these two mechanisms is presented.

17.2.1 Membrane receptors activated by SCFAs

G protein-coupled receptors (GPCRs) constitute a family of membrane proteins characterized by a common motif, the seven-transmembrane helix. These receptors are activated by a variety of ligands pertaining to different chemical classes such as amino acids (i.e. basic L-amino acids activate GPCR6A), carbohydrates (i.e. T1R2/T1R3 receptors are activated by ligands such as glucose and fructose), ions (i.e. extracellular calcium-sensing receptor, CaR) and lipids (most of the eicosanoid receptors).

In 1997, a new class of GPCRs was isolated (Sawzdargo *et al.*, 1997). Later, these GPCRs were shown to be activated by fatty acids: long-chain fatty acids, including the most abundant plasma fatty acids, palmitic, stearic and oleic acids, are ligands of GPR40 (Briscoe *et al.*, 2003; Kotarsky *et al.*, 2003), while the

SCFAs activate the GPR41 and GPR43 receptors (Brown *et al.*, 2003; Itoh *et al.*, 2003; Nilsson *et al.*, 2003). GPR41 (also known as free fatty acid receptor 3 or FFAR3) and GPR43 (free fatty acid receptor 2 or FFAR2) differ in tissue expression and function: GPR41, which couples exclusively to the pertussis toxin-sensitive Gi/o protein, has a more widespread expression pattern than GPR43, being expressed in adipose tissue, pancreas, spleen, lymph nodes, bone marrow, and peripheral blood mononuclear cells (Brown *et al.*, 2003; Le Poul *et al.*, 2003). GPR43 couples to both Gi/o and Gq proteins and is expressed by leukocytes, particularly neutrophils and monocytes, and adipocytes (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003; Zaibi *et al.*, 2010).

Binding of agonists (SCFAs or synthetic agonists, phenylacetamide-1 and -2) to GPR41 and GPR43 activates several intracellular pathways, which are integrated and lead to cellular responses. Phospholipase C (Gq) and adenylate cyclase (Gi) are involved in the intracellular signalling of GPR43 receptor. Other pathways, such as activation of small G-proteins (Rac/Cdc42), mitogen-activated protein kinases (p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2)) and even transcription factors (i.e. activating transcriptional factor-2, also known as ATF-2) (Maslowski *et al.*, 2009), seem also to be important for the effects of the SCFAs on cells (i.e. activation of p38 has an essential role in the chemotactic effects of SCFAs on neutrophils) (Sina *et al.*, 2009) (see Plate II in colour section between pages 516 and 517).

17.2.2 Inhibition of HDAC activity

The eukaryotic genome is found wrapped in the nucleus in proteins called histones. Modifications on these proteins, such as acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines, and ubiquitination and sumoylation of lysines, modify the expression of several genes.

SCFAs are inhibitors of HDACs, a group of enzymes found in the nucleus and cytoplasm that, together with the histone acetyltransferases (HATs), control the degree of histone acetylation. Inhibitors of HDAC activity increase the acetylation of both histone and non-histone proteins such as transcription factors (i.e. nuclear factor kappa (NFκB), MyoD, p53 and N-FAT), cytoskeletal proteins (i.e. α-tubulin) and molecular chaperones (i.e. Hsp90) (Glozak *et al.*, 2005) (Plate II). Through this action, SCFAs modulate gene transcription; i.e. inhibition of HDAC activity (using butyrate) in HT29 colonic epithelial cells modulated the expression of a total of more than 1900 genes (approximately, 10% of the total gene set analysed) (Daly and Shirazi-Beechey, 2006).

17.3 Effect of short-chain fatty acids on leukocyte function

Several studies have been performed *in vitro* in order to investigate the effects of SCFAs on cells involved in inflammation and immune responses. In this section, we discuss separately some of the studies conducted in isolated neutrophils, macrophages/monocytes and lymphocytes.

17.3.1 Neutrophils

Neutrophils are the first cells that migrate into tissues in response to invading bacteria and other micro-organisms, and are the predominant infiltrating cell types in the cellular phase of the acute inflammatory response. These cells are recruited and migrate from the bloodstream to inflamed tissue in a multistep and highly controlled process (Ley *et al.*, 2007).

The effect of SCFAs on recruitment of neutrophils has, in particular, attracted the attention of several groups. In part, this reflects the interest in understanding the actions of these compounds in the GI tract and at sites of anaerobic bacterial infections such as periodontal tissue and abscess, where concentrations of 10–50 mM of SCFAs have been described (Ladas *et al.*, 1979; Rotstein *et al.*, 1985). In this context, SCFAs induce chemotaxis of neutrophils (Le Poul *et al.*, 2003; Maslowski *et al.*, 2009; Sina *et al.*, 2009; Vinolo *et al.*, 2009b), an effect dependent on activation of GPR43 receptor, and modulate the expression of adhesion molecules on neutrophils (Vinolo *et al.*, 2009b) and endothelial cells (Zapolska-Downar *et al.*, 2004; Miller *et al.*, 2005; Zapolska-Downar and Naruszewicz, 2009), indicating that these compounds may be important for neutrophil migration.

Once in the inflammatory site, neutrophils internalize, kill and digest bacteria and fungi through effector mechanisms such as production of reactive oxygen species (ROS) and release of granule enzymes. SCFAs affect the phagocytic capacity of neutrophils, but both inhibition and stimulation of neutrophil phagocytosis by SCFAs have been described (Eftimiadi *et al.*, 1990; Mills *et al.*, 2006; Vinolo *et al.*, 2009a). The effects of SCFAs on the production of ROS by neutrophils are also inconsistently reported: some groups have found that SCFAs increase ROS production (Stringer *et al.*, 1996; Nakao *et al.*, 1998), while others have found inhibition (Eftimiadi *et al.*, 1987; Tonetti *et al.*, 1991; Liu *et al.*, 2001; Sandoval *et al.*, 2007; Vinolo *et al.*, 2009a). These discrepancies probably result from differences in the protocols used such as concentration of SCFAs, measurement of ROS with different methodologies (i.e. lucigenin-amplified chemiluminescence and reduction of cytochrome c), stimulus used to elicit ROS production (i.e. PMA or fMLP), source and state of neutrophil activation (i.e. neutrophils isolated from human blood or elicited rat neutrophils).

Neutrophils are important sources of cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-8 (the rat analogue is CINC-2 $\alpha\beta$) and transforming growth factor (TGF)- β (Cassatella, 1995) that, together with other mediators such as nitric oxide (NO) and eicosanoids, are released in the inflammatory site and coordinate different steps of the inflammatory process. SCFAs modify the production of inflammatory mediators by neutrophils: Tedelind *et al.* (2007) have shown that acetate, propionate and butyrate at 30 mM reduce TNF- α production by lipopolysaccharide (LPS)-stimulated human neutrophils. We have found that SCFAs (propionate and butyrate) inhibit the production of pro-inflammatory mediators (TNF- α , CINC-2 $\alpha\beta$ and NO) by rat neutrophils (Vinolo *et al.*, 2011). In agreement with previous studies performed in macrophages (Park *et al.*, 2007; Usami *et al.*, 2008) and other cell types such as endothelial cells (Ogawa *et al.*, 2003; Zapolska-Downar *et al.*, 2004), we have found that propionate and butyrate

inhibit NF κ B activation (Vinolo *et al.*, 2011). Our hypothesis is that by inhibiting HDAC activity and thus modifying the acetylation status of proteins, SCFAs modulate the activation of transcription factors such as NF κ B and consequently the expression of genes involved in the inflammatory response.

The migration of neutrophils to inflammatory foci, production of inflammatory mediators and destruction of micro-organism are modulated by SCFAs. Both activation (i.e. induction of chemotaxis) and inhibition (i.e. attenuation of cytokine production) of neutrophil functions were observed in *in vitro* studies. These contradictory findings make it difficult to know clearly what the *in vivo* effect of these fatty acids might be. For example, in periodontal disease, a condition in which there is large amounts of SCFAs along with chemotactic factors, other activators of neutrophils and the bacteria, it is not yet known what effect the SCFAs are having. Also, whether SCFAs contribute to evasion of bacteria from the immune system or facilitate bacterial destruction by phagocytes remains to be defined.

17.3.2 Macrophages/monocytes

Macrophages are resident phagocytic cells found in almost all the tissues. These cells are formed after monocyte differentiation and are present with distinct specializations in different tissues such as lung (alveolar macrophages), central nervous system (microglia) and liver (Kupffer cells). In addition to an important role in tissue homeostasis, macrophages have a pivotal role in different steps of the immune and inflammatory responses such as microbial recognition, antigen presentation, recruitment of cells and resolution of the inflammatory process.

The differentiation and maturation of monocytes to macrophages and dendritic cells is regulated by a variety of factors such as cytokines (i.e. *in vitro*, macrophage colony-stimulating factor (M-CSF) is required to macrophage differentiation, while dendritic cell differentiation is promoted by granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4) and cell–cell interaction. As already shown for adipocytes (Yoo *et al.*, 2006), erythroid (Andersson *et al.*, 1979) and muscle cells (Iezzi *et al.*, 2002), butyrate modulates the differentiation of monocytes (Millard *et al.*, 2002). This fatty acid partially prevents the cytokine-driven differentiation of monocytes to macrophages and to dendritic cells *in vitro*, and inhibits their functionality (i.e. their phagocytic capacity and ability to stimulate T-cell response) (Millard *et al.*, 2002; Wang *et al.*, 2008).

Macrophages are the major source of inflammatory mediators, which are linked to different pathologies such as insulin resistance and diabetes, atherosclerosis, rheumatoid arthritis and neurodegenerative diseases (Kinne *et al.*, 2000; Boyle, 2005; Griffin, 2006; Olefsky and Glass, 2010). Once activated, these cells produce large amounts of cytokines (i.e. TNF- α , IL-1 β and IL-6), chemokines (i.e. CC-chemokine ligand-2 (CCL2)), nitric oxide (NO) and arachidonic acid derivatives (i.e. thromboxane A₂ and prostaglandins E₂ and F_{1 α}). Several groups have shown that SCFAs modulate the production of some of these mediators by macrophages (Table 17.1).

Table 17.1 Effect of SCFAs on the production of inflammatory mediators by macrophages

Cell type	SCFA	Effect on production of mediators	Reference
RAW 264.7 cells	Butyrate	↓ TNF- α , IL-6, NO ↑ IL-10	Park <i>et al.</i> , 2007, Chakravorty <i>et al.</i> , 2000
Blood mono-nuclear cells	Butyrate	↓ TNF- α , ↑ PGE ₂	Usami <i>et al.</i> , 2008
Monocytes and macrophages	Butyrate Phenylbutyrate	↓ TNF- α ↓ TNF- α , IL-6	Fukae <i>et al.</i> , 2005 Grabiec <i>et al.</i> , 2010
Monocytes	Butyrate	↓ TNF- α , IL-12, IFN- γ ↑ IL-10	Saemann <i>et al.</i> , 2000
Microglial cells	Acetate, propionate and butyrate	↓ MCP-1, IL-10 ↑ PGE ₂	Cox <i>et al.</i> , 2009
	Propionate and butyrate	↑ IL-6, NO	Huuskonen <i>et al.</i> , 2004
	Butyrate	↓ TNF- α , IL-6, NO	Park <i>et al.</i> , 2005
Rat primary microglia	Butyrate	↓ NO	
Murine BV2 cell line	Butyrate	↓ NO	
Kupffer cells	Butyrate	↓ TNF- α , ↓ PGE ₂	Perez <i>et al.</i> , 1998

IFN- γ , interferon- γ ; IL-6, interleukin-6; MCP, macrophage chemoattractant protein; NO, nitric oxide; PG, prostaglandin; TNF, tumour necrosis factor.

↑ increase; ↓ decrease.

In general, SCFAs, mainly butyrate, have anti-inflammatory effects on macrophages: they prevent the production of pro-inflammatory cytokines (i.e. TNF- α) and increase the secretion of anti-inflammatory cytokines such as IL-10 (Table 17.1), although this latter effect was not observed in all studies.

Inhibition of HDAC activity by SCFAs and modulation of different signalling pathways such as NF κ B and ERK seem to be the mechanisms involved in the effects of SCFAs on macrophages (Chakravorty *et al.*, 2000; Park *et al.*, 2007; Usami *et al.*, 2008).

Microglial cells are resident immune cells of the central nervous system (CNS). Activation of these cells leading to production of several inflammatory mediators such as cytokines and NO is a defence reaction of the CNS to insults (Nakamura, 2002). Chronic or excessive activation of these cells has detrimental effects to the CNS and seems to be involved in the initiation and progression of several neurodegenerative diseases (i.e. Alzheimer's disease and Parkinson's disease). Most of the studies performed with butyrate have shown attenuation of microglial activation (Park *et al.*, 2005; Chen *et al.*, 2007), an effect that probably occurs due to inhibition of HDAC activity. So, butyrate or other inhibitors of HDAC may be useful in reducing inflammation in the CNS as already demonstrated by Kim *et al.* (2007). This group has shown that butyrate, valproic acid and trichostatin A (all

inhibitors of HDAC) exert antineuroinflammatory and neuroprotective effects in the ischaemic brain of rats (Kim *et al.*, 2007).

The importance of the acetylation status of histone and non-histone proteins in macrophage function was highlighted in a recent study. The authors have shown that, by using a compound that mimics acetylated histones (I-BET), there is a reduction in the expression of inflammatory genes (Nicodeme *et al.*, 2010). A protection against LPS-induced endotoxic shock and bacterial-induced sepsis was also found (Nicodeme *et al.*, 2010).

17.3.3 Lymphocytes

Lymphocytes are responsible for the adaptive immune response. These cells display receptors on their membrane surface which recognize a broad range of non-self-antigens and allow them to generate specific responses with the aim of eliminating invading pathogens and infected or tumoural cells. Several subsets of lymphocytes have been described such as natural killer cells, B- and T-lymphocytes, which can be further divided in other subtypes such as T-helper, T-cytotoxic and T-regulatory.

SCFAs modify various lymphocyte functions:

- **T-cell proliferation:** butyrate inhibits lymphocyte proliferation in response to stimuli (i.e. concanavalin-A and immobilized anti-CD3 monoclonal antibody) (Bohmig *et al.*, 1997; Cavaglieri *et al.*, 2003).
- **Production of cytokines:** incubation of lymphocytes with butyrate reduces the production of IL-2, a cytokine that stimulates growth, differentiation and survival of antigen-selected T-lymphocytes, and interferon- γ (IFN- γ), which has immunomodulatory actions, being particularly important in the response to viral infection, tumour cells and in autoimmune conditions, after stimulation with concanavalin-A or anti-CD3 and anti-CD8 (Cavaglieri *et al.*, 2003; Selma Dagtas and Gilbert, 2010). On the other hand, butyrate promotes the production of IL-10, an anti-inflammatory cytokine, by lymphocytes (Cavaglieri *et al.*, 2003).

Considering the fact that dendritic cells and macrophages play a central role in the establishment of the immune responses, the suppressive effect of butyrate on these cells, together with the inhibition of T-lymphocyte responses, indicate that butyrate or its derivatives may be therapeutically used as immunosuppressive agents.

Several studies have also indicated that SCFAs may modulate inflammation and immune responses not only by affecting the function of leukocytes, but also by inducing apoptosis of the cells involved in these processes (i.e. lymphocytes (Kurita-Ochiai *et al.*, 2001; Bailon *et al.*, 2010), macrophages (Ramos *et al.*, 2002) and neutrophils (Aoyama *et al.*, 2010)). The mechanism by which SCFAs, particularly butyrate and propionate, induce apoptosis involves inhibition of HDAC activity and activation of caspases (i.e. caspases 3, 8 and 9) but not the GPR43 receptor (Kurita-Ochiai *et al.*, 2001; Ramos *et al.*, 2002; Bailon *et al.*, 2010).

17.4 Effect of short-chain fatty acids on inflammation

Considering the potential anti-inflammatory actions of SCFAs, several studies have attempted to use them or their derivatives on various inflammatory conditions *in vivo* in human and animal models (Tables 17.2 and Table 17.3). Herein, we present a brief description of the findings of some of the studies focused on the use of SCFAs in the treatment of inflammatory bowel disease (IBD) (Table 17.2), sepsis and ischaemic tissue injury (Table 17.3).

17.4.1 Inflammatory bowel disease

IBD, characterized by chronic and spontaneous inflammation of the GI tract, can be divided in two main diseases: ulcerative colitis (UC) and Crohn's disease (CD). The main difference between these pathologic conditions is the site of the gut affected. The pathological damage caused by the inflammatory process in UC is only seen in the mucosa of the large bowel, whereas in CD any part of the gut can be affected (Fiocchi, 1998). Despite the advances in the understanding of IBD, the aetiology of this disease remains unclear. Any exacerbated inflammatory response of the gut in reaction to a luminal agent may upregulate the synthesis and release of pro-inflammatory mediators, such as cytokines and ROS, which initiate and perpetuate the inflammatory process in the intestine (Sartor, 1997).

SCFAs, mainly butyrate, are important in maintaining the health and integrity of the colonic mucosa since these fatty acids are the predominant form of fuel for colonocytes, and modulate gene expression and proliferation of these cells. Some studies have indicated that in IBD patients the concentrations of SCFAs within the gut lumen are reduced, suggesting a relationship between the concentration of these fatty acids and IBD (Vernia *et al.*, 1988; Huda-Faujan *et al.*, 2010). Other studies have shown that not only the levels of SCFAs may be altered in IBD, but also their uptake (Thibault *et al.*, 2007) and/or oxidation (Chapman *et al.*, 1994). Patients with IBD have diminished levels of the monocarboxylate transporter 1 (MCT1), a protein involved in the butyrate uptake by colonocytes, which consequently reduces the intracellular availability of butyrate (Thibault *et al.*, 2007). Considering these observations, diverse forms of intervention that lead to increased SCFA concentrations in the GI tract, such as ingestion of dietary fibre, use of enemas or oral administration of sodium butyrate have been tested in *in vivo* studies (Vernia *et al.*, 1995, 2000, 2003; Tarrerias *et al.*, 2002; Di Sabatino *et al.*, 2005; Hamer *et al.*, 2010; Huda-Faujan *et al.*, 2010).

Dietary fibres are not hydrolysed by mammalian digestive enzymes. This group includes non-starch polysaccharides, resistant starch, cellulose, hemicelluloses, oligosaccharides, pectins, gums, lignin and waxes (James *et al.*, 2003). Although these substances constitute the same group, they do not share the same characteristics of solubility. Some of them are soluble and are then highly fermentable by colonic bacteria, producing SCFAs and promoting bacterial growth. On the other hand, the insoluble dietary fibres are barely fermentable leading to a small production of SCFAs (Galvez *et al.*, 2005). The difference in fibre solubility plays a key role in the treatment of IBD due to the production of SCFAs.

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Table 17.2 Effects of SCFAs or their precursors in intestinal inflammation

Model	Intervention	Effects observed	Reference
Rat; TNBS-induced colitis	Diet with resistant starch	Improvement of symptoms; epithelial cell proliferation; regeneration of laminin; growth of intestinal bacteria	Jacobasch <i>et al.</i> , 1999
Rat; DSS-induced colitis	Diet with fructo-oligosaccharides or resistant starch	Resistant starch improved gut macroscopically and histologically; fructo-oligosaccharides had no effect	Moreau <i>et al.</i> , 2003
BALB/c mice; DSS-induced colitis	Diet with cellobiose	Reduction of weight loss; diminished tissue oedema; attenuation of inflammatory cytokine concentrations	Nishimura <i>et al.</i> , 2010
Rat; HLA-B27 transgenic	Diet with added fibre	Reduction in myeloperoxidase and nitric oxide synthase activities; restoration of colonic glutathione levels; diminished TNF- α concentrations	Rodriguez-Cabezas <i>et al.</i> , 2002
Rat; HLA-B27 transgenic Mice; DSS-induced colitis	Diet with germinated barley foodstuff	Suppression of NF κ B activity; reduction of IL-8 production	Kanauchi <i>et al.</i> , 1999, Kanauchi <i>et al.</i> , 2003
Rat; DSS-induced colitis	Diet with inulin <i>versus</i> inulin enemas	Lower lesion score; decreased inflammatory mediators; lower tissue myeloperoxidase activity	Videla <i>et al.</i> , 2001
Rat; TNBS-induced colitis	SCFA enemas	Increased the duration of pain in rats with colitis	Tarrerias <i>et al.</i> , 2002
Human; distal UC	Sodium butyrate enemas (100 mM)	Improved clinical symptoms and inflammatory scores	Scheppach <i>et al.</i> , 1992
Human; UC patients	Sodium butyrate enemas (100 mM)	Minor effects on colonic inflammation and oxidative stress; increased IL-10/IL-12 ratio and CCL5 concentrations	Hamer <i>et al.</i> , 2010
Human; UC patients	Topical 5-ASA + sodium butyrate	Improvement versus the baseline; only one remission	Vernia <i>et al.</i> , 2003

DSS, dextran sodium sulphate; IL, interleukin; NF κ B, nuclear factor kappa B; RS, resistant starch; SCFAs, short-chain fatty acids; TNF, tumour necrosis factor, TNBS, trinitrobenzenesulfonic acid; UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid; CCL5, chemokine CC-ligand 5.

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Table 17.3 Effects of SCFAs in models of total parenteral nutrition and critical illness

Model	Intervention	Effects observed	Reference
Rats on TPN	Intravenous versus intra-caecal infusion of SCFAs	Both treatments reduced mucosal atrophy associated with TPN	Koruda <i>et al.</i> , 1990
Children on TPN	Intravenous SCFAs	Reduced mucosal atrophy associated with TPN; reduced catheter infection	McAndrew <i>et al.</i> , 1999
Rats; CLP-induced sepsis	Intravenous sodium butyrate (500 mg/kg body weight)	Reduced serum alanine aminotransferase, creatinine and pulmonary myeloperoxidase activity; improved survival	Zhang <i>et al.</i> , 2007
BALB/c mice with lipo-polysaccharide-induced acute lung injury	Oral butyrate (10 mg/kg body weight)	Reduced lung histopathology, alveolar haemorrhage and neutrophil infiltration into lungs	Ni <i>et al.</i> , 2010
Rats with acute lung injury	Intraperitoneal administration of trichostatin (2 mg/kg body weight) or sodium butyrate (200 mg/kg body weight)	Reduced neutrophil infiltration and ICAM-1 and E-selectin expression in lung	Zhang <i>et al.</i> , 2010
Rats with ischaemia-induced injury	Intraperitoneal sodium butyrate (300 mg/kg body weight)	Reduced infarct size; diminished brain infarct volume and microglial activation	Kim <i>et al.</i> , 2007

CLP, caecal ligation puncture; ICAM-1, intercellular adhesion molecule; SCFAs, short chain fatty acids; TPN, total parenteral nutrition.

The interest in the use of fibres in the treatment of IBD patients started with the study of Davies and Rhodes (1978). Probably, due to the type of fibre used (mainly insoluble), the authors did not find any beneficial effect of this intervention in UC patients. However, other studies subsequently showed improvement in the IBD patients, such as normalization of the intestinal transit (Hallert *et al.*, 1991), restoration of intestinal mucosa (Jacobasch *et al.*, 1999; Moreau *et al.*, 2003) and reduction in colonic oedema (Nishimura *et al.*, 2010) after ingestion of soluble fibres. A potential mechanism that could underlie these effects is the utilization of butyrate by colonic epithelial cells (Jacobasch *et al.*, 1999; Rodriguez-Cabezas *et al.*, 2002). The aerobic production of adenosine triphosphate (ATP) after butyrate oxidation by colonocytes (Jacobasch *et al.*, 1999) re-establishes the metabolic function, regenerates the inflamed colonic tissue and preserves the integrity of the colonic mucosa (MacDermott, 1996). The improvement in the metabolic function is one, but not the unique, mechanism involved in the beneficial effects of SCFAs in IBD patients. Rodriguez-Cabezas *et al.* (2002, 2003) observed that the ingestion of fibres inhibits the production and release of inflammatory mediators, such as cytokines in HLA-B27 transgenic rats and in trinitro-benzenesulfonic acid-induced colitis in rats. Other studies, in experimental models of colitis, also indicated that dietary fibres can reduce the production of pro-inflammatory mediators such as IL-6, IL-8 and TNF- α (Kanauchi *et al.*, 1999, 2003; Videla *et al.*, 2001). In addition to anti-inflammatory properties, SCFAs also modulate oxidative stress through an increase in the colonic glutathione (GSH) levels (Rodriguez-Cabezas *et al.*, 2002).

The use of SCFA enemas is another alternative strategy for the treatment of IBD. Scheppach *et al.* (1992) and Vernia *et al.* (1995) observed that rectal administration of butyrate improves clinical symptoms and overall inflammatory scores in humans. However, other groups failed to show beneficial effects of this type of treatment in IBD patients (Hamer *et al.*, 2010) or in experimental models using rats (Tarrerias *et al.*, 2002). The topical application of butyrate and mesalazine (5-ASA), a known anti-inflammatory drug, improved the efficacy of treatment in patients with active UC and refractory distal UC, indicating that such combinations may be more effective than the SCFAs or the anti-inflammatory (5-ASA) alone (Vernia *et al.*, 2003).

Most of the studies indicate that the use of SCFAs or substances that lead to endogenous production of SCFAs (such as dietary fibres) has beneficial effects in IBD (Table 17.2). More studies are necessary to establish in which stage of IBD the use of SCFAs is more beneficial, the frequency of administration, the amount to give, the form of administration and the association of these fatty acids with other drugs.

17.4.2 Sepsis

Sepsis is a condition defined by the presence of both infection and an inflammatory systemic response (Levy *et al.*, 2003) and is characterized by excessive production of inflammatory mediators such as TNF- α , IL-1 and high-mobility group box

(HMGB1), which lead to injury in different tissues and multiple-organ dysfunction. Despite significant advances in the field of sepsis management, severe sepsis (sepsis with organ dysfunction) and septic shock (condition characterized by the presence of a hypotension despite adequate volume resuscitation, in the absence of other causes for hypotension) still present today mortality rates of 25–30% and 40–70%, respectively.

Considering the fact that butyrate, as highlighted before, has important anti-inflammatory actions, some studies tested whether this fatty acid could have beneficial effects in sepsis (Zhang *et al.*, 2007; Weber and Kerr, 2008; Ni *et al.*, 2010). In a model of sepsis induced by caecal ligation and puncture (CLP) in rats, the administration of sodium butyrate (500 mg/kg, i.v.) prevented damage to liver, kidneys and lung, and improved survival (Zhang *et al.*, 2007). The authors correlated these effects with a reduction in the production of HMGB1, a mediator secreted by activated monocytes, macrophages and neutrophils, which triggers the release of several pro-inflammatory cytokines (Wang *et al.*, 1999) and seems to be a sufficient and necessary mediator for severe sepsis (Ulloa and Tracey, 2005).

Promising results were also obtained with the use of butyrate for reducing lung injury caused by sepsis (Ni *et al.*, 2010; Zhang *et al.*, 2010). Acute lung injury (ALI) is characterized by the loss of the alveolar-capillary barrier integrity and refractory hypoxaemia, which occurs due to direct or indirect lung insults (Ware and Matthay, 2000). Sepsis is the most usual cause of ALI (Balibrea and Arias-Diaz, 2003). The pretreatment with sodium butyrate significantly alleviated septic lung injury and attenuated histological lesions in mice (Zhang *et al.*, 2010). Reductions in leukocyte infiltration and pulmonary myeloperoxidase (MPO) activity were observed in mice treated with sodium butyrate intraperitoneal injection (200 mg/kg), an effect that may be related to the inhibition of HDAC (Zhang *et al.*, 2010).

In another study, in which ALI was induced in mice by intratracheal instillation of LPS, the oral administration of butyrate (10 mg/kg) reduced the concentrations of TNF- α , IL-1 β and NO in bronchoalveolar lavage fluid (Ni *et al.*, 2010). These results were associated with a suppressive action of butyrate on expression of NF κ B subunit p65. Reduction in alveolar haemorrhage and infiltration of neutrophils were observed indicating a protective effect of butyrate in this condition (Ni *et al.*, 2010).

Another possible application of butyrate is as a component of parenteral nutrition. The major characteristic of parenteral nutrition is the direct delivery of nutrients into the circulatory system without passing through the liver (Shils *et al.*, 2005). In spite of the importance of this procedure in ill patients, total parenteral nutrition (TPN) has serious side effects such as gut mucosal atrophy and bacterial translocation that are the main causes of central catheter infection and bacterial spreading in the organism. Considering this problem, McAndrew *et al.* (1999) studied the effects of SCFA added into TPN and observed an improvement in mucosal atrophy and reduction in bacterial translocation in rats. Other groups obtained the same results using two different forms of butyrate administration (intraecal and intravenous) indicating that the form of administration does not

significantly influence the effects of SCFAs on intestinal mucosa (Koruda *et al.*, 1990).

17.4.3 Ischaemia tissue injury

Sodium butyrate and other inhibitors of HDAC (valproic acid and trichostatin A) have been used with the aim of preventing tissue injury induced by ischaemia (Kim *et al.*, 2007). The authors of this study reported that, in rats subjected to cerebral artery occlusion, post-insult treatment with butyrate reduced brain infarct volume and suppressed microglial activation and expression of inflammatory markers in the ischaemic brain (Kim *et al.*, 2007). According to the authors, multiple mechanisms are involved in stroke and, since there is no effective treatment for this disease, butyrate should be tested in clinical trials.

17.5 Perspectives of short-chain fatty acids and inflammation

In our view, three points will be of great interest in this research field in the next few years:

1. To understand how the gut microbiota and their products such as the SCFAs regulate the development of the immune system and its association with several diseases such as diabetes, IBD and obesity. Several recent studies have contributed to explaining this interaction (Cani and Delzenne, 2009; Gao *et al.*, 2009; De Filippo *et al.*, 2010; Rabot *et al.*, 2010; Schwartz *et al.*, 2010), but further studies are required.
2. SCFAs are produced during infection by anaerobic bacteria. In spite of the fact that we already know that these compounds modulate the function and death of leukocytes, little information is available about the *in vivo* effects of SCFAs on bacteria survival and host response. The understanding of the role of SCFAs in infection will allow the development of strategies to block the production of SCFAs or their cellular effects in order to facilitate the elimination of pathogenic bacteria by the immune system.
3. SCFAs are able to modulate immune cell function and most of the studies performed *in vivo* (i.e. in experimental models of sepsis and in IBD patients), mainly with butyrate, present promising results. The development of new therapeutical strategies based on these compounds or their cellular targets (i.e. inhibition of HDAC activity or activation of GPR41 and 43) should be focus of research.

17.6 Sources of further information

There is a vast literature available on SCFAs and inflammation/immune function. The book *Physiological and Clinical Aspects of Short-Chain Fatty Acids* edited by

Cummings, Rombeau and Sakata (2004) is a comprehensive and well-written collection of information on SCFAs and should be read by those who want to further investigate this issue. The review 'Regulation of inflammation by short chain fatty acids' published in *Nutrients* (2011) covers many of the points discussed in this chapter in further detail.

17.7 Acknowledgement

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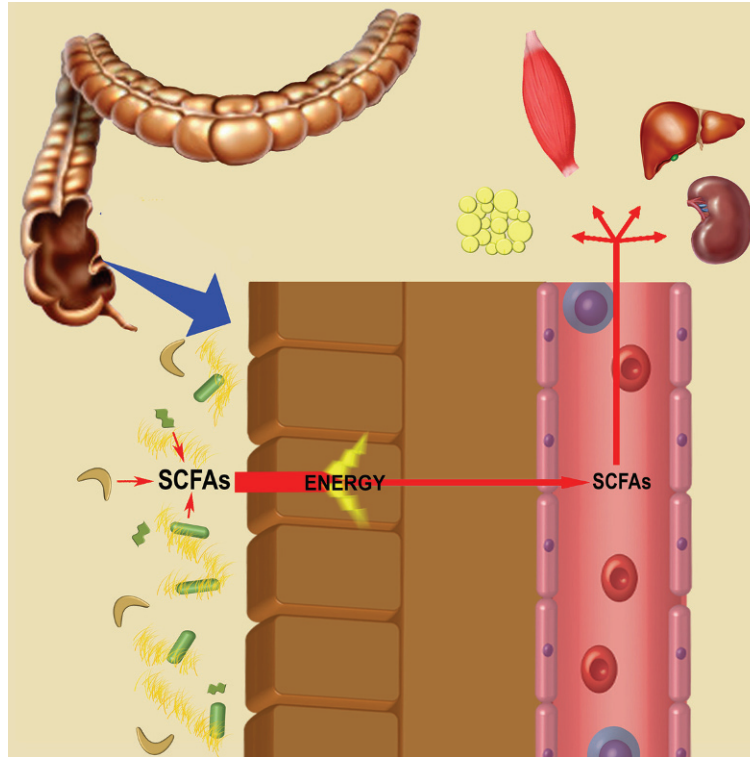


Plate I (Chapter 17) Schematic representation of the production, absorption and handling of SCFAs. Acetate, propionate and butyrate are produced in the intestine by anaerobic bacterial fermentation of fibres. These fatty acids are then absorbed into and utilized in different tissues, particularly in the colonic epithelium and the liver.

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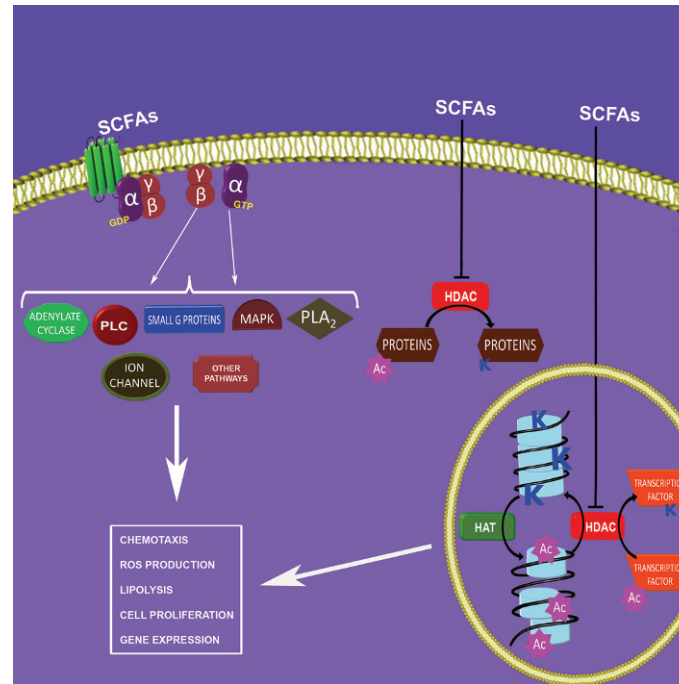


Plate II (Chapter 17) Summary of the main mechanisms by which SCFAs act on cells. Schematic overview of the signalling pathways activated downstream to GPR41 and 43 receptors and representation of the effects of SCFAs through inhibition of histone deacetylase (HDAC) activity. GPR41 couples to G_i and GPR43 couples to G_i and G_q proteins. Both interact with several enzymes such as adenylate cyclase, phospholipase C (PLC), small G proteins (i.e. Rac and Rho), mitogen-activated protein kinases (MAPK) and phospholipase A₂ (PLA₂), ion channels and transcription factors. SCFAs may also act on cells through inhibition of HDAC. This class of enzymes, together with histone acetylases (HAT), controls the acetylation state of histones and non-histone proteins. By inhibiting HDAC activity, SCFAs modify functions of several proteins and transcription of different genes.

18

Long-chain fatty acids and inflammatory processes

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Abstract: This chapter discusses the influence of dietary long-chain fatty acids on aspects of inflammation including markers of the inflammatory process and functional responses of inflammatory cells. It discusses the underlying mechanisms by which fatty acids can act and places the observations made in the context of treatment of chronic inflammatory diseases.

Key words: cytokine, eicosanoid, resolvin, fish oil, leukocyte.

18.1 Introduction

For the purpose of this chapter, long-chain fatty acids are considered to be those with chain lengths of 12 or more carbons. Long chain fatty acids occur naturally in the human diet; they circulate in the bloodstream either as components of more complex lipids (e.g. triglycerides, phospholipids, cholesteryl esters) within lipoproteins or ‘free’ (i.e. as non-esterified fatty acids); they are stored within adipose tissue (as triglycerides); and they are present in cell membranes, playing an important structural and functional role (Calder and Burdge, 2004). The diet, bloodstream, adipose tissue, and cell membranes contain a number of different fatty acids, each present in different concentrations and relative amounts. Table 18.1 lists long-chain fatty acids of dietary or physiological significance. Fatty acids within the body can come preformed from the diet or can be synthesized within the body from non-lipid precursors or from other fatty acids (Fig. 18.1). Fatty acids have a generic structure: a hydrocarbon chain terminated by a methyl group at one end of the chain and a carboxyl group, often esterified, at the other. However,

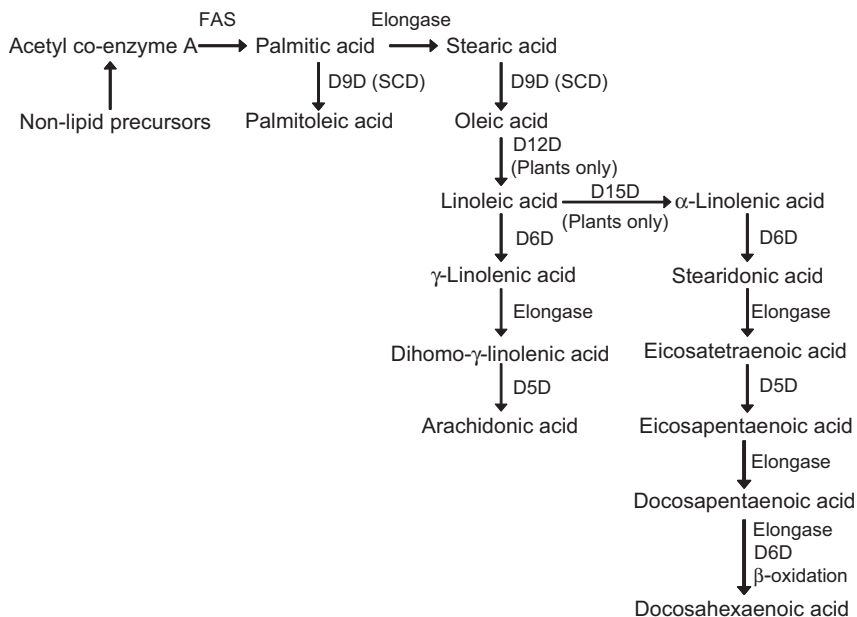


Fig. 18.1 The pathway of biosynthesis of fatty acids. DXD, delta-X desaturase; FAS, fatty acid synthase; SCD, stearoyl CoA desaturase.

particular structural characteristics distinguish different families of long-chain fatty acids. These characteristics are:

- the absence or presence of double bonds within the hydrocarbon chain (distinguishing saturated and unsaturated fatty acids);
- the presence of a single or multiple double bonds within the hydrocarbon chain (distinguishing monounsaturated and polyunsaturated fatty acids);
- the configuration of the double bonds within the hydrocarbon chain (distinguishing *cis* and *trans* fatty acids);
- the position of the double bonds within the hydrocarbon chain (distinguishing different omega families of fatty acids; the omega classification refers to the carbon on which the first double bond is found, counting the methyl terminus carbon as number 1).

Fatty acids have both systematic and common names (Table 18.1). They are also described by a shorthand nomenclature which denotes the number of carbons and double bonds in the hydrocarbon chain and, in the case of unsaturated fatty acids, the omega classification (Table 18.1). Figure 18.1 shows the metabolic relationships among the different long-chain fatty acids, while Table 18.1 lists their names and typical dietary sources. Two fatty acids, linoleic (18:2n-6) and α -linolenic (18:3n-3; ALNA), cannot be synthesized in humans and are termed essential fatty acids. Because the composition of the diet differs amongst different people, there is significant variation in the intake of individual long-chain fatty

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Table 18.1 Long-chain fatty acid nomenclature and common dietary sources

Systematic name	Trivial name	Shorthand notation	Abbreviation	Common sources
Dodecanoic	Lauric	12:0		Milk; coconut oil
Tetradecanoic	Myristic	14:0		Milk
Hexadecanoic	Palmitic	16:0		Milk; eggs; animal fats; meat; cocoa butter; palm oil (other vegetable oils contain lesser amounts); fish oils
Octadecanoic	Stearic	18:0		Milk; eggs; animal fats; meat; cocoa butter
<i>cis</i> 9-hexadecenoic	Palmitoleic	16:1n-7		Desaturation of palmitic acid; fatty fish; fish oils
<i>cis</i> 9-octadecenoic	Oleic	18:1n-9		Desaturation of stearic acid; milk; eggs; animal fats; meat; cocoa butter; most vegetable oils, especially olive oil
<i>trans</i> 9-octadecenoic	Elaidic	–		Biohydrogenation of vegetable oils
<i>trans</i> 11-octadecenoic	Trans vaccenic	–		Milk (ruminant); animal (ruminant) fats
<i>cis</i> 9, <i>cis</i> 12-octadecadienoic	Linoleic	18:2n-6		Cannot be synthesized in animals; some milks; eggs; animal fats; meat; most vegetable oils, especially corn, sunflower, safflower and soybean oils; green leaves
<i>cis</i> 9, <i>trans</i> 11-octadecadienoic	9, 11 Conjugated linoleic acid	–	CLA	Milk (ruminant); meat (ruminant)

(Continued)

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Table 18.1 Continued

Systematic name	Trivial name	Shorthand notation	Abbreviation	Common sources
All <i>cis</i> 9, 12, 15-octadecatrienoic	α -Linolenic	18:3n-3	ALNA	Cannot be synthesized in animals; green leaves; some vegetable oils, especially rapeseed, soybean and flaxseed oils; flaxseeds; some nuts
All <i>cis</i> 6, 9, 12-octadecatrienoic	γ -Linolenic	18:3n-6	GLA	Synthesized from linoleic acid; borage and evening primrose oils
All <i>cis</i> 6, 9, 12, 15-octatetraenoic	Stearidonic acid	18:4n-3		Synthesized from ALNA; echium oil; genetically modified soybean oil
All <i>cis</i> 8, 11, 14-eicosatrienoic	Dihomo- γ -linolenic	20:3n-6	DGLA	Synthesized from GLA
All <i>cis</i> 5, 8, 11, 14-eicosatetraenoic	Arachidonic	20:4n-6	ARA	Synthesized from linoleic acid via GLA and DGLA; meat; milk; some algal oils; fish oils
All <i>cis</i> 5, 8, 11, 14, 17-eicosapentaenoic	Eicosapentaenoic	20:5n-3	EPA	Synthesized from ALNA; seafood, especially fatty fish; fish oils
All <i>cis</i> 7, 10, 13, 16, 19-docosapentaenoic	Docosapentaenoic	22:5n-3	DPA	Synthesized from ALNA via EPA; seafood, especially fatty fish; fish oils
All <i>cis</i> 4, 7, 10, 13, 16, 19-docosahexaenoic	Docosahexaenoic	22:6n-3	DHA	Synthesized from ALNA via EPA and DHA; seafood, especially fatty fish; fish oils; some algal oils

acids within the population. However, in most western diets saturated fatty acids and oleic acid (18:1n-9) are consumed in the greatest amounts (several tens of g per day), followed by linoleic acid (about 10 g per day) and then ALNA (about 1 g per day). Very long-chain (i.e. ≥ 20 carbon) highly unsaturated fatty acids are consumed in much lower amounts, typically tens to hundreds of mg per day.

18.2 Importance of fatty acid composition for function of cells involved in inflammation

Fatty acids are vital structural and functional components of phospholipids in all cell membranes. They contribute to the physical state of the membrane ensuring the appropriate environment for the function of membrane proteins such as receptors, transporters, ion channels, enzymes and so on. A change in the physical state of the membrane, which may accompany a change in its fatty acid composition, can influence the function of membrane proteins (Brenner, 1984; Stubbs and Smith, 1984; Murphy, 1990). Membrane structures, termed rafts, form upon cell stimulation, and bring together proteins involved in the relevant signal transduction pathway, so forming signalling platforms (Simons and Toomre, 2000; Pike, 2003). It appears that the fatty acid composition of the cell membrane can influence raft formation and so can influence signalling processes (Yaqoob, 2009; Yaqoob and Shaikh, 2010). Several membrane phospholipids are precursors of molecules involved in signal transduction, such as diacylglycerols, and the fatty acid composition of the signalling molecule, which reflects that of the parent phospholipid, can influence its biological activity and potency (Miles and Calder, 1998). Fatty acids released from cell membrane phospholipids are precursors for mediators involved in inflammation such as prostaglandins (PGs) and leukotrienes (LTs). Thus, there are a number of mechanisms by which altering the fatty acid composition of the membranes of cells involved in inflammation can influence the functional responses of those cells.

The phospholipids of monocytes, neutrophils and lymphocytes taken from the blood of humans consuming typical western diets, contain a lot of palmitic (16:0), oleic and linoleic acids, about 1–2% of fatty acids as dihomo- γ -linoleic acid (20:3n-6; DGLA), 10–20% as arachidonic acid (20:4n-6; ARA), with about 0.5–1% eicosapentaenoic acid (20:5n-3; EPA) and about 1.5–3% docosahexaenoic acid (22:6n-3; DHA) (Lee *et al.*, 1985; Endres *et al.*, 1989; Sperling *et al.*, 1993; Caughey *et al.*, 1996; Healy *et al.*, 2000; Yaqoob *et al.*, 2000; Kew *et al.*, 2003, 2004; Rees *et al.*, 2006). There are, however, marked differences in fatty acid contents of the different phospholipid classes (Sperling *et al.*, 1993).

The fatty acid composition of human inflammatory cells can be modified by changed intakes of various n-6 and n-3 fatty acids. γ -linolenic acid (18:3n-6; GLA) does not accumulate in significant amounts in inflammatory cell membranes, even when its intake is increased (Johnson *et al.*, 1997; Rosetti *et al.*, 1997; Yaqoob *et al.*, 2000). Instead, the content of its derivative DGLA is increased (Johnson *et al.*, 1997; Rosetti *et al.*, 1997; Yaqoob *et al.*, 2000). Increased intake of ARA results

in increased ARA in blood mononuclear cells, a mixture of lymphocytes and monocytes (Thies *et al.*, 2001a). Increased intake of ALNA results in increased EPA in mononuclear cells (Kelley *et al.*, 1993; Caughey *et al.*, 1996; Kew *et al.*, 2003) and neutrophils (Mantzioris *et al.*, 1994; Healy *et al.*, 2000). The ALNA derivative stearidonic acid also results in increased EPA in mononuclear cells (Miles *et al.*, 2004). Many studies have demonstrated that increased intake of EPA+DHA, usually from fish oil supplements, results in a higher content of EPA and DHA in mononuclear cells and neutrophils (Lee *et al.*, 1985; Endres *et al.*, 1989; Chilton *et al.*, 1993; Sperling *et al.*, 1993; Caughey *et al.*, 1996; Healy *et al.*, 2000; Yaqoob *et al.*, 2000; Thies *et al.*, 2001a; Kew *et al.*, 2003, 2004; Rees *et al.*, 2006; Browning *et al.*, 2012). The increased content of EPA and DHA is associated with a decreased content of n-6 fatty acids, including ARA. Time-course studies suggest that the net incorporation of EPA and DHA into human inflammatory cells begins within days and reaches its peak within a few weeks (Healy *et al.*, 2000; Yaqoob *et al.*, 2000; Thies *et al.*, 2001a; Rees *et al.*, 2006; Faber *et al.*, 2011). Studies that have used multiple doses of fish oil show that the incorporation of EPA and DHA occurs in a dose–response manner (Healy *et al.*, 2000; Rees *et al.*, 2006; Browning *et al.*, 2012).

18.3 Lipid mediators produced from n-6 and n-3 fatty acids

Eicosanoids are a family of lipid mediators synthesized from 20-carbon polyunsaturated fatty acids (PUFAs), and include PGs, thromboxanes and LTs. Because inflammatory cells usually have a high membrane content of ARA, ARA is the usual precursor for eicosanoid synthesis (Fig. 18.2). ARA is released from the phospholipids through the action of phospholipase A_2 enzymes, which are activated by inflammatory stimuli. The free ARA then acts as a substrate for cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 enzymes. COX enzymes lead to PGs and thromboxanes, LOX enzymes lead to LTs, and cytochrome P450 enzymes to hydroxyeicosatetraenoic and epoxyeicosatrienoic acids (Fig. 18.2). ARA yields the 2-series PGs and the 4-series LTs, both families being important mediators and regulators of inflammation (Lewis *et al.*, 1990; Tilley *et al.*, 2001; Kroetz and Zeldin, 2002). For example, PGE_2 increases vascular permeability, vasodilation, blood flow and local pyrexia, and potentiates pain caused by other agents. It also promotes the production of some matrix metalloproteinases that can cause local tissue damage and destruction (e.g. to bone in rheumatoid arthritis). LTB_4 increases vascular permeability, enhances local blood flow, is a potent chemotactic agent for leukocytes, induces release of lysosomal enzymes and enhances release of reactive oxygen species and inflammatory cytokines. Because of the association between these eicosanoids and inflammation, their synthesis and action are targets for a range of non-specific and specific pharmaceuticals, including aspirin and COX-2 inhibitors.

Endocannabinoids are complex eicosanoids produced by cleavage of phospholipids by phospholipases (Di Marzo, 2008). Arachidonoyl ethanolamide

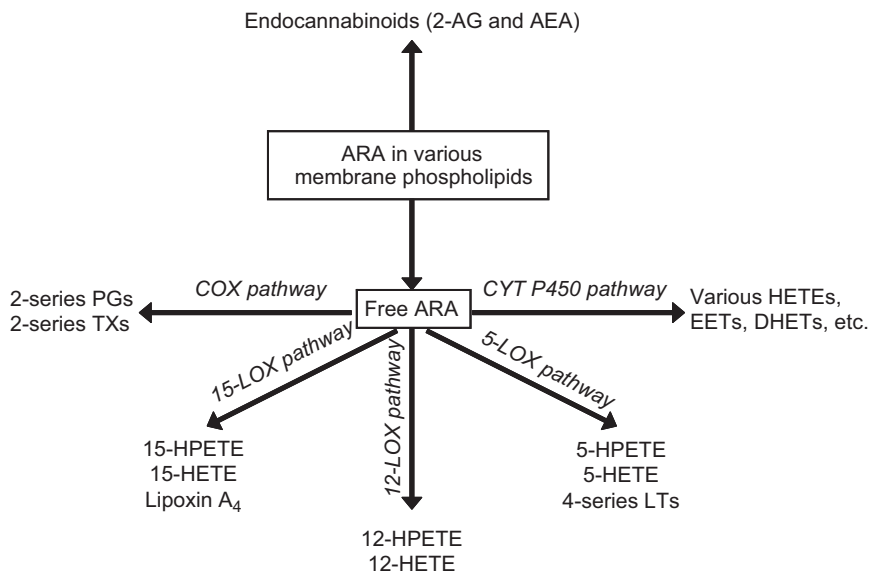


Fig. 18.2 Outline of the pathways of eicosanoid synthesis from arachidonic acid. AEA, arachidonoyl ethanolamine (anandamide); 2-AG, 2-arachidonoyl glycerol; ARA, arachidonic acid; COX, cyclooxygenase; CYT P450, cytochrome P450 enzymes; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane.

(AEA), also known as anandamide, and 2-arachidonoyl glycerol (2-AG) are the two main ARA-containing endocannabinoids. AEA is formed by a pair of reactions involving conversion of phosphatidylethanolamine to N-acyl-phosphatidylethanolamine followed by the action of phospholipase D. 2-AG is formed as a result of the sequential actions of phospholipase C and a diacylglycerol lipase (Di Marzo, 2008).

Although it is generally considered that eicosanoids derived from ARA are pro-inflammatory, as indicated above, this is an over-simplification (Calder, 2009). For example, PGE₂ inhibits the production of two of the classic pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukin (IL)-1, by monocytes and macrophages (see Calder, 2009). Furthermore, in more recent years it has been discovered that PGE₂ inhibits 5-LOX and so decreases production of the inflammatory 4-series LTs (Levy *et al.*, 2001) and induces 15-LOX so promoting the formation of lipoxins (Levy *et al.*, 2001; Vachier *et al.*, 2002). These lipoxins have been found to have anti-inflammatory effects (Gewirtz *et al.*, 2002). These findings show that PGE₂ possesses both pro- and anti-inflammatory actions; this may apply to other eicosanoids too. There may be a temporal change in the role of PGE₂ with an initial pro-inflammatory effect followed by a role in resolution of inflammation through inhibition of 5-LOX and induction of lipoxin production.

DGLA is a substrate for COX, 5-LOX and 15-LOX giving rise to 1-series PGs, 3-series LT, and 15-hydroxy-DGLA, respectively. PGE₁ has several anti-

inflammatory effects including inhibition of TNF, IL-1 and IL-6 production by macrophages (see Calder and Zurier, 2001). 15-hydroxy-DGLA is an inhibitor of 5-LOX and 12-LOX and so can decrease the synthesis of inflammatory mediators from ARA. Supplementing the human diet with GLA (more than about 2.5 g per day) increased PGE₁ production and decreased PGE₂, LTB₄ and LTC₄ production (Johnson *et al.*, 1997; Wu *et al.*, 1999).

Several studies have described decreased production of PGE₂ and 4 series-LTs by human inflammatory cells following use of fish oil supplements for a period of weeks to months (Lee *et al.*, 1985; Endres *et al.*, 1989; Meydani *et al.*, 1991; Sperling *et al.*, 1993; von Schacky *et al.*, 1993; Caughey *et al.*, 1996). These studies have usually used fairly high intakes of the marine n-3 PUFAs, most often several grams per day. Kelley *et al.* (1999) demonstrated that DHA at a high dose (6 g/day) decreased PGE₂ and LTB₄ production by lipopolysaccharide (LPS)-stimulated mononuclear cells. A diet rich in ALNA also results in decreased production of ARA-derived eicosanoids (Caughey *et al.*, 1996). This is probably because ALNA can be converted to EPA, resulting in a higher EPA and lower ARA content of eicosanoid-producing cells.

Because EPA is a 20-carbon highly unsaturated fatty acid it is also a substrate for the COX, LOX and cytochrome P450 enzymes that produce eicosanoids, and EPA- and DHA-containing phospholipids are substrates for the synthesis of endocannabinoids. However, the mediators produced from EPA and DHA have a different structure from those made from ARA (e.g. PGE₃ rather than PGE₂ and LTB₅ rather than LTB₄). The functional significance of generation of eicosanoids from n-3 fatty acids is that they are often much less biologically active than those produced from ARA (Goldman *et al.*, 1983; Lee *et al.*, 1984; Bagga *et al.*, 2003). One reason for this reduced biological potency is that eicosanoid receptors typically have a much lower affinity for the EPA-derived mediator than for the ARA-derived one (Wada *et al.*, 2007). Thus, EPA results in decreased production of potent eicosanoids from ARA and increased production of weak eicosanoids. EPA- and DHA-containing endocannabinoids have anti-inflammatory properties in cell culture systems (Balvers *et al.*, 2010; Meijerink *et al.*, 2011).

Resolvins are a family of lipid mediators produced from EPA (E-series) and DHA (D-series). Protectins are produced from DHA; these are also referred to as neuroprotectins when generated within neural tissue. The synthesis of resolvins and protectins involves the COX and LOX pathways, with different epimers being produced in the presence and absence of aspirin (Serhan *et al.*, 2000a,b, 2002, 2008). The biological effects of resolvins and protectins have been examined extensively in cell culture and in animal models of inflammation, where they have been shown to be anti-inflammatory and inflammation resolving. For example, resolvins D1 and E1 and protectin D1 all inhibited transendothelial migration of neutrophils, preventing the infiltration of neutrophils into sites of inflammation; resolvin D1 inhibited IL-1 β production; and protectin D1 inhibited TNF and IL-1 β production (Serhan *et al.*, 2000a, 2002, 2008). Resolvins reduce inflammation and exert protection in experimental animals in models of inflammatory disease

including arthritis (Lima-Garcia *et al.*, 2011), colitis (Arita *et al.*, 2005) and asthma (Aoki *et al.*, 2008; Haworth *et al.*, 2008).

18.4 Effects of specific fatty acids on inflammation

18.4.1 Overview

Human studies investigating the impact of altered fatty acid exposures on inflammation have used two different general approaches. The first has been to examine the relationship between intake or status of a particular fatty acid and a marker of inflammation. The second approach has been to increase intake of the fatty acid under study and to identify the effect of changed intake or status. This latter approach has often used fatty acids supplied as supplements, usually in capsules. The markers of inflammation studied in these investigations have fallen into two types. The first is blood markers of inflammation such as white cell count, white cell activation state or serum or plasma concentrations of acute-phase proteins, cytokines or soluble adhesion molecules. The second is the response of white cells to an *ex vivo* stimulus such as bacterial endotoxin (LPS), the outcome usually being the production of one or more mediators involved in inflammation (e.g. TNF).

Saturated fatty acids

In vitro studies show that inflammatory cells can be activated directly by saturated fatty acids, especially lauric acid (12:0), in a process involving the LPS receptor, Toll-like receptor (TLR)-4, and subsequent activation of the pro-inflammatory transcription factor nuclear factor kappa B (NFκB) (Lee *et al.*, 2001; Weatherill *et al.*, 2005). Fernandez-Real *et al.* (2003) did not see any relationship between serum saturated fatty acids and C-reactive protein (CRP) or IL-6 concentrations in lean humans. However, in overweight individuals serum saturated fatty acids were positively associated with IL-6 concentration and the ratio of saturated fatty acids to n-6 or n-3 fatty acids was positively associated with IL-6 and CRP concentrations, respectively. A study in overweight adolescents showed positive relationships between total saturated fatty acids in plasma phospholipids or cholesteryl esters and IL-6, but not CRP, concentration (Klein-Platat *et al.*, 2005). Thus, there is general agreement between two studies in overweight humans that saturated fatty acid exposure is associated with higher IL-6 concentration, but lean individuals seem not to show this relationship. An intervention study feeding diets rich in stearic acid (18:0) or in the combination of lauric, myristic (14:0) and palmitic acids to men for five weeks showed higher concentrations of CRP, fibrinogen, IL-6 and soluble E-selectin (SE-selectin) compared with a diet enriched in oleic acid (Baer *et al.*, 2004). There are few other intervention studies chronically increasing saturated fatty acid intake in humans and reporting inflammatory markers. However, the data that exists from two observational and one intervention study indicates that saturated fatty acids may increase inflammation.

Trans fatty acids

Data from the Nurses' Health Study revealed positive relationships between dietary trans fatty acid intake and concentrations of six inflammatory markers, including CRP, IL-6 and three soluble adhesion molecules (Lopez-Garcia *et al.*, 2005). In a five week intervention study in healthy men, a *trans* fatty acid-enriched diet resulted in higher CRP and IL-6 concentrations than diets rich in oleic acid, stearic acid or the combination of lauric, myristic and palmitic acids (Baer *et al.*, 2004). Furthermore, the concentration of sE-selectin was higher than in all other dietary groups including the stearic acid and lauric+myristic+palmitic groups. Thus, one association study and one intervention study suggest that dietary *trans* fatty acids elevate the concentrations of a range of inflammatory markers, including CRP, IL-6 and adhesion molecules, and that *trans* fatty acids may be more 'pro-inflammatory' than saturated fatty acids.

Conjugated linoleic acids

In vitro and animal feeding studies have suggested enhanced inflammation with increased conjugated linoleic acid (CLA) exposure (Li *et al.*, 2005, 2006; Moloney *et al.*, 2007). However, human studies using CLA-rich capsules provide mixed findings (Riserus *et al.*, 2002; Tricon *et al.*, 2004; Nugent *et al.*, 2005; Ramakers *et al.*, 2005; Smedman *et al.*, 2005; Song *et al.*, 2005; Mullen *et al.*, 2007). Two studies reported that CLA, especially the *trans*-10, *cis*-12 isomer, increases CRP concentration, but not the concentrations of several cytokines or soluble adhesion molecules (Riserus *et al.*, 2002; Smedman *et al.*, 2005). However, a number of other studies have failed to show an effect of CLA on CRP concentration (Tricon *et al.*, 2004; Ramakers *et al.*, 2005; Mullen *et al.*, 2007). Most studies have used mixtures of CLA isomers in different proportions, and this may explain the discrepancy in the literature since the precise dose of the more potent isomer (perhaps the *trans*-10, *cis*-12 isomer) differs amongst these studies. The two studies showing increased CRP with CLA provided 2.1 g (Smedman *et al.*, 2005) and 2.7 g (Riserus *et al.*, 2002) per day *trans*-10, *cis*-12 CLA. The studies showing no effect of CLA on CRP used between 0.4 and 2.5 g per day of this isomer (Tricon *et al.*, 2004; Ramakers *et al.*, 2005; Mullen *et al.*, 2007). Studies investigating the effect of several weeks of intake of CLA on *ex vivo* inflammatory cytokine production by mononuclear cells report little or no effect (Kelley *et al.*, 2001; Albers *et al.*, 2003; Nugent *et al.*, 2005; Ramakers *et al.*, 2005). Thus, it is not yet clear whether CLA *per se* or specific CLA isomers increase inflammation.

Linoleic acid

Because of the role of linoleic acid as the precursor of ARA, which is, in turn, the substrate for synthesis of inflammatory eicosanoids like PGE₂ and 4-series LTs, it is widely considered that elevated n-6 and low n-3 fatty acids (i.e. a high n-6 to n-3 fatty acid ratio) in the diet will promote inflammation. However, available evidence does not seem to support this idea. Dietary intakes of linoleic acid were not associated with CRP or IL-6 concentrations in subgroups of the Physicians' Health Study and the Nurses' Health Study (Pischon *et al.*, 2003), while the

concentration of linoleic acid in blood lipids (Klein-Platat *et al.*, 2005; Ferrucci *et al.*, 2006) or granulocytes (Madsen *et al.*, 2001) was not associated with CRP or IL-6 concentration. In contrast, a large Swedish study reported an inverse association between linoleic acid in cholesteryl esters and CRP concentration (Petersson *et al.*, 2008). Total n-6 fatty acids in serum fatty acids in overweight, but not in lean, subjects were inversely associated with IL-6, but not CRP, concentration (Fernandez-Real *et al.*, 2003). The ratio of saturated fatty acids to n-6 fatty acids in serum lipids or in plasma phospholipids was positively associated with IL-6, but not CRP, in overweight subjects (Fernandez-Real *et al.*, 2003; Klein-Platat *et al.*, 2005). This suggests that decreasing saturated fatty acid status while increasing n-6 fatty acid status might reduce inflammation.

Several intervention studies with ALNA (see below) have involved a group consuming a high intake of linoleic acid, frequently as the control for the high ALNA intake. These studies provide some information about the impact of linoleic acid on inflammation. Rallidis *et al.* (2003, 2004) and Paschos *et al.* (2007) provided 11 g linoleic acid per day to subjects habitually consuming about 11 g linoleic acid per day, approximately doubling intake. This did not alter the concentrations of CRP, TNF, IL-6, serum amyloid A, sICAM-1 or sE-selectin, but the concentration of sVCAM-1 was decreased. In the study of Yaqoob *et al.* (2000) one group of subjects consumed about 6 g per day linoleic acid from safflower oil capsules for 12 weeks; dietary intake was not reported, but average habitual intake of linoleic acid among adults in the UK is about 11 g per day. Thus these individuals increased intake of linoleic acid by about 55%. There was no effect on production of TNF, IL-1 α , IL-1 β or IL-6 by LPS-stimulated mononuclear cells. These studies did not alter any aspect of diet but required subjects to consume oil providing linoleic acid on top of the normal diet. They show that markedly increasing linoleic acid intake in those consuming on average about 11 g per day does not increase inflammation.

Arachidonic acid

ARA is the most prevalent n-6 fatty acid and polyunsaturated fatty acid in the membranes of inflammatory cells and other cells involved in inflammation such as endothelial cells and platelets. This reflects the important functional role of ARA as a precursor of eicosanoids, as described earlier. Since eicosanoids are classically associated with inflammatory processes and are targeted by common anti-inflammatory therapies, it is generally considered that ARA itself will enhance inflammation. However, observations that classical pro-inflammatory mediators like PGE₂ can also exert anti-inflammatory effects and that ARA gives rise to anti-inflammatory mediators like lipoxin A₄, have started to challenge the earlier view (Calder, 2009). Indeed, there was no association between ARA in granulocytes and CRP concentration (Madsen *et al.*, 2001), while serum free ARA was not associated with soluble intercellular adhesion molecule-1 (sICAM-1) or sE-selectin concentrations and was actually inversely associated with soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration (Yli-Jama *et al.*, 2002). Ferrucci *et al.* (2006) reported no association between ARA in plasma and CRP,

TNF, IL-1 β , IL-10 and soluble IL-6 receptor (sIL-6R) concentrations, while there was an inverse association with IL-6 and IL-1 receptor antagonist (ra) concentrations and a positive association with transforming growth factor (TGF)- β concentration. These observations suggest either that plasma ARA has little impact on inflammation (does not affect CRP or TNF) or that it is anti-inflammatory (lowers IL-6; increases TGF- β).

There are very few intervention studies with ARA reporting on inflammation. In an uncontrolled study, Kelley *et al.* (1997) reported higher granulocyte numbers in the blood of a small group of healthy men taking a supplement of 1.5 g per day ARA for 100 days, while there was no effect on production of TNF, IL- β or IL-6 by LPS-stimulated mononuclear cells. In another small, but controlled, study, older subjects consumed capsules providing 700 mg per day ARA for 12 weeks (Thies *et al.*, 2001b); there was no effect on plasma sVCAM-1, sICAM-1 or sE-selectin concentrations or on production of TNF, IL- β or IL-6 by LPS-stimulated mononuclear cells. Collectively, these data suggest little impact of modest increases of ARA intake on inflammation. The effect of lowering ARA intake below habitual levels on inflammation has not been investigated.

α -linolenic acid

Increased consumption of ALNA results in an increased content of EPA in the membranes of inflammatory cells (Kelley *et al.*, 1993; Mantzioris *et al.*, 1994; Caughey *et al.*, 1996; Healy *et al.*, 2000; Kew *et al.*, 2003) in parallel with a decreased content of ARA. This might be expected to affect inflammation. However, dietary ALNA intake was not associated with CRP or IL-6 concentrations in one study on subgroups of the Physicians' Health Study and the Nurses' Health Study (Pischon *et al.*, 2003). In a second study on another subgroup of the Nurses' Health Study, ALNA intake was not associated with CRP, sICAM-1 or sE-selectin concentrations but was associated with lower IL-6 and sVCAM-1 concentrations (Lopez-Garcia *et al.*, 2004). The concentration of ALNA in blood lipids (Klein-Platat *et al.*, 2005; Petersson *et al.*, 2008) or granulocytes (Madsen *et al.*, 2001) was not associated with CRP or IL-6 concentration. In another study, there was no association between ALNA in plasma fatty acids and several cytokines including IL-6 and TNF, but there was an inverse association with CRP (Ferrucci *et al.*, 2006). These association studies suggest a limited anti-inflammatory effect of ALNA *per se*.

Several intervention studies have involved high ALNA intakes, usually by providing flaxseed oil in capsules or in liquid form or foodstuffs made using flaxseed oil. Frequently, these studies have used a control group with a high intake of linoleic acid, with the comparison essentially being replacement of linoleic acid with ALNA. These studies have produced inconsistent findings with some identifying effects of ALNA on some markers and not on others (Thies *et al.*, 2001b; Rallidis *et al.*, 2003, 2004; Bemelmans *et al.*, 2004; Paschos *et al.*, 2004; Zhao *et al.*, 2004), and some studies finding no effects (Junker *et al.*, 2001; Paschos *et al.*, 2007). However, what is apparent is that a substantial increase in intake of ALNA can decrease inflammation as indicated by circulating CRP, IL-6 or soluble

adhesion molecules (Thies *et al.*, 2001b; Rallidis *et al.*, 2003, 2004; Bemelmans *et al.*, 2004; Paschos *et al.*, 2004; Zhao *et al.*, 2004).

Caughey *et al.* (1996) reported that 13.7 g per day ALNA for four weeks resulted in decreased production of TNF and IL-1b by LPS-stimulated mononuclear cells by 27 and 30%, respectively. By comparison, fish oil providing EPA+DHA at 2.7 g per day decreased the production of these two inflammatory cytokines by 70 and 78%, respectively (Caughey *et al.*, 1996). Thus, on a gram per day basis, marine n-3 fatty acids are about ten times more potent than ALNA. In contrast to the observations of Caughey *et al.* (1996), several studies using lower intakes of ALNA (2–9.5 g per day) did not find effects on neutrophil chemotaxis, neutrophil or monocyte respiratory burst, or TNF, IL-1b and IL-6 production by LPS-stimulated mononuclear cells (all studied *ex vivo*), or on soluble adhesion molecule concentrations (Healy *et al.*, 2000; Thies *et al.*, 2001b; Kew *et al.*, 2003; Wallace *et al.*, 2003). Taken together, these data suggest that a marked increase in ALNA intake is required in order for anti-inflammatory effects to be seen. Even then, the effects will be much more modest than those exerted by marine n-3 PUFA. The observed effects of high intakes of ALNA on inflammation seem to be due to its derivative EPA rather than to ALNA itself (Zhao *et al.*, 2004). Thus, the likely explanation for the lack of anti-inflammatory effect of ALNA in many studies is that there has been insufficient conversion to the more active EPA.

Marine n-3 fatty acids

Incorporation of EPA and DHA into inflammatory cells is partly at the expense of ARA, resulting in less substrate available for synthesis of the classic inflammatory eicosanoids. Through altered eicosanoid production, marine n-3 fatty acids could affect inflammation and inflammatory processes, although they also exert non-eicosanoid-mediated actions on cell signalling and gene expression (Fig. 18.3). These actions include modification of formation of signalling platforms (i.e. rafts) in the cell membrane, activation of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor (PPAR)- γ and inhibition of activation of the pro-inflammatory transcription factor NF κ B (Calder, 2011, 2012). Recent studies indicate a membrane-bound G protein-coupled receptor called GPCR120 that is important to the anti-inflammatory effects of DHA, and perhaps other unsaturated fatty acids, in macrophages (Oh *et al.*, 2010). The effects of marine n-3 fatty acids have been examined in many model systems, and findings from cell culture systems and from animal models are generally consistent in identifying anti-inflammatory actions (Calder, 2003). Furthermore, clinical trials have demonstrated anti-inflammatory effects and some clinical benefit from fish oil administration in diseases with a frank inflammatory basis including rheumatoid arthritis (RA), inflammatory bowel diseases (IBD) and childhood asthma (Calder, 2006).

Several cross-sectional studies show an inverse association between intake or status of marine n-3 fatty acids and markers of inflammation. Data from subgroups of the Physicians' Health Study and the Nurses' Health Study showed inverse associations between dietary intake of EPA+DHA and concentrations of CRP

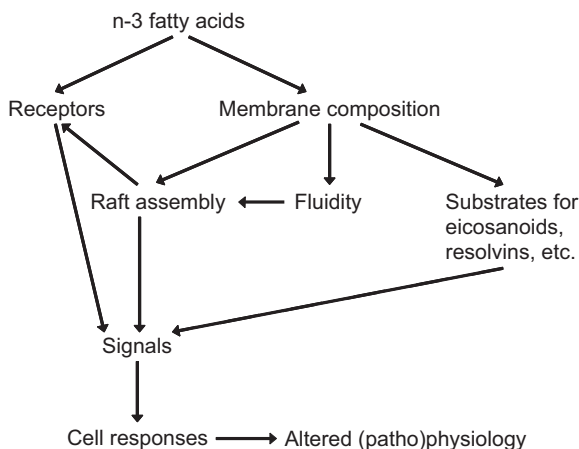


Fig. 18.3 Overview of the mechanisms by which n-3 fatty acids can influence inflammation. Modified from *European Journal of Pharmacology*, Vol. 668, P.C. Calder, Fatty acids and inflammation: the cutting edge between food and pharma, pp. S50–S58, Copyright (2011), with permission from Elsevier.

(Pischon *et al.*, 2003) and CRP, sICAM-1, sVCAM-1 and sE-selectin (Lopez-Garcia *et al.*, 2004). The concentration of either EPA or DHA in granulocyte membranes was inversely associated with CRP concentration in one study (Madsen *et al.*, 2001); the effect of DHA was stronger than that of EPA. Serum non-esterified EPA and DHA were both inversely associated with concentrations of sVCAM-1 and sICAM-1 in patients at risk of coronary heart disease (Yli-Jama *et al.*, 2002); EPA was also inversely associated with sE-selectin concentration. Plasma cholesteryl ester EPA was inversely associated with CRP concentration in overweight subjects (Klein-Platat *et al.*, 2005). In another study, plasma EPA was inversely associated with IL-6 concentration and positively associated with the concentrations of the anti-inflammatory cytokines IL-10 and TGF- β (Ferrucci *et al.*, 2006). Furthermore, plasma DHA was inversely associated with IL-6 and TNF concentrations and was also positively associated with the concentrations of IL-10 and TGF- β (Ferrucci *et al.*, 2006). Thus, observational studies suggest that both EPA and DHA are anti-inflammatory.

The ready availability of fish oil capsules has facilitated numerous supplementation studies of marine n-3 fatty acids. Studies have shown that these fatty acids lower the concentrations of CRP (Ciubotaru *et al.*, 2003; Browning *et al.*, 2007; Rasic-Milutinovic *et al.*, 2007), IL-6 (Ciubotaru *et al.*, 2003; Browning *et al.*, 2007; Rasic-Milutinovic *et al.*, 2007), TNF (Rasic-Milutinovic *et al.*, 2007), IL-18 (Troseid *et al.*, 2009), sICAM-1 (Eschen *et al.*, 2004; Hjerkin *et al.*, 2005; Yamada *et al.*, 2008; Yusof *et al.*, 2008), sVCAM-1 (Thies *et al.*, 2001b; Yamada *et al.*, 2008) and sE-selectin (Abe *et al.*, 1998). Both EPA and DHA (4 g per day for six weeks) decreased plasma TNF concentrations in type 2 diabetics, although DHA was more potent (Mori *et al.*, 2003).

Dietary supplementation studies using between 3.1 and 14.4 g per day of EPA+DHA have demonstrated decreased chemotaxis of human neutrophils and monocytes towards various chemoattractants (Lee *et al.*, 1985; Endres *et al.*, 1989; Schmidt *et al.*, 1989, 1991, 1992; Luostarinen *et al.*, 1992; Sperling *et al.*, 1993). One study reported decreased expression of ICAM-1 on the surface of monocytes after intake of 1.5 g per day EPA+DHA for several weeks (Hughes *et al.*, 1996). Supplementation studies providing 3.1–8.4 g per day of EPA+DHA reported decreased production of oxidants, including superoxide or hydrogen peroxide, by stimulated human neutrophils and monocytes (Fisher *et al.*, 1990; Thompson *et al.*, 1991; Varming *et al.*, 1995; Luostarinen and Saldeen, 1996). Fish oil providing more than 2 g per day of EPA+DHA decreased the production of TNF, IL-1 and IL-6 by mononuclear cells in some studies in healthy volunteers (Endres *et al.*, 1989; Meydani *et al.*, 1991; Caughey *et al.*, 1996; Trebble *et al.*, 2003). Caughey *et al.* (1996) reported a significant inverse correlation between the EPA content of mononuclear cells and the ability of those cells to produce TNF and IL-1 β in response to LPS. Kelley *et al.* (1999) showed that 6 g per day of DHA for 12 weeks decreased production of TNF and IL-1 β by LPS-stimulated mononuclear cells. There are, however, a number of such studies reporting no effects of EPA+DHA on *ex vivo* cytokine production (see Calder, 2006).

Thus, EPA+DHA, when given at sufficient doses, are anti-inflammatory, although findings from different studies are not entirely consistent. This lack of consistency may be related to differences in: duration of treatment; sample size; characteristics of the populations studied (e.g. age, healthy vs. diseased, type of disease, smokers vs non-smokers); background diet; dose of EPA+DHA used; relative contribution of EPA and DHA, since they may have different anti-inflammatory potencies; chemical formulation (e.g. triglyceride vs ethyl ester); and degree of oxidative stress present. One other factor is genetic differences among individuals, which may have an impact on the ability of n-3 fatty acids to exert an anti-inflammatory effect. This was first identified by Grimble *et al.* (2002) who showed that the ability of fish oil to lower the LPS-stimulated production of TNF by blood mononuclear cells was determined in part by polymorphisms within the TNF and lymphotoxin genes.

The ability of marine n-3 fatty acids to downregulate several aspects of inflammation suggests that these fatty acids might be important in determining the development and severity of inflammatory diseases and that they may be useful as a component of therapy. Amongst the classic inflammatory conditions, fish oil has been most thoroughly examined in RA (Calder 2008a). Animal models have demonstrated that marine n-3 fatty acids can delay the onset of arthritis, reduce its severity and improve joint pathology (Leslie *et al.*, 1985; Volker *et al.*, 2000; Ierna *et al.*, 2010). Cleland *et al.* (2006) found that RA patients using fish oil supplements were more likely to reduce use of non-steroidal anti-inflammatory drugs (NSAIDs) and to be in remission than those patients who did not use fish oil. Randomized controlled trials of fish oil in RA report improvements in several clinical outcomes including reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced joint pain, reduced time to fatigue, increased

grip strength and decreased use of NSAIDs (Calder, 2008a). The dose of n-3 fatty acids used in these trials has typically been high, between about 1 and 7 g per day and averaging about 3.5 g per day. This dose would be difficult to achieve through the diet, but can be achieved through use of supplements or liquid oil. A meta-analysis concluded that dietary fish oil supplementation for three months significantly reduced tender joint count and morning stiffness (Fortin *et al.*, 1995). A more recent meta-analysis concluded that fish oil reduces patient-assessed joint pain, duration of morning stiffness, number of painful and/or tender joints and consumption of NSAIDs (Goldberg and Katz, 2009). Thus there is fairly robust evidence of the efficacy of marine n-3 fatty acids in RA.

Animal models have demonstrated that marine n-3 fatty acids decrease chemically-induced colonic damage and inflammation (see Calder, 2008b). The effects on disease severity were, in all cases, associated with a reduction in production of ARA-derived eicosanoids. A more recent study investigated chemically-induced colitis in *fat-1* mice (Hudert *et al.*, 2006). The mice showed much less colonic damage and inflammation than wild-type mice, and this was associated with a marked change in the pattern of inflammatory mediators present in colonic tissue. A study in IL-10 knock-out mice that spontaneously develop colitis demonstrated reduced colonic inflammation if the mice were fed fish oil (Chapkin *et al.*, 2007). EPA and DHA are incorporated into gut mucosal tissue of patients with IBD who supplement their diet with fish oil, and this is associated with reduced inflammation (see Calder, 2008b). Some randomized controlled trials of fish oil in IBD have reported clinical benefits including improved clinical score, improved gut mucosal histology, improved sigmoidoscopic score, lower rate of relapse and decreased use of corticosteroids (see Calder, 2008b). The dose of EPA+DHA used in these trials has typically been high, between 2.5 and 6 g per day and averaging about 4 g per day. However, a number of trials do not report benefits and meta-analyses are not supportive of an effect (MacLean *et al.*, 2004; De Ley *et al.*, 2007; Turner *et al.*, 2007, 2009). Thus, despite some favourable studies, there is at best only weak evidence that marine n-3 fatty acids have clinical benefits in human IBD.

ARA-derived eicosanoids are produced by the cells that mediate pulmonary inflammation in asthma and are believed to be major mediators of asthmatic bronchoconstriction. DHA reduced lung inflammation and improved lung function in a murine model of asthma (Yokoyama *et al.*, 2000). Epidemiologic data link high n-6 fatty acid or low n-3 fatty acid consumption with increased risk of developing childhood asthma. Studies have reported anti-inflammatory effects of fish oil in patients with asthma, such as decreased production of 4-series LTs and reduced leukocyte chemotaxis (see Calder, 2006). However, randomized controlled trials of fish oil in adult asthma have reported no clinical benefit (Calder, 2006). Conversely, marine n-3 fatty acids may improve lung function in children with asthma (Hodge *et al.*, 1998; Nagakura *et al.*, 2000) and may improve disease activity (Nagakura *et al.*, 2000). A meta-analysis identified that there was no consistent effect of fish oil on lung function, asthma symptoms or asthma medication use, but stated that one study in children showed improved lung function and reduced asthma medication use (Thien *et al.*, 2002). Clearly, more needs to be done in this area, especially in children.

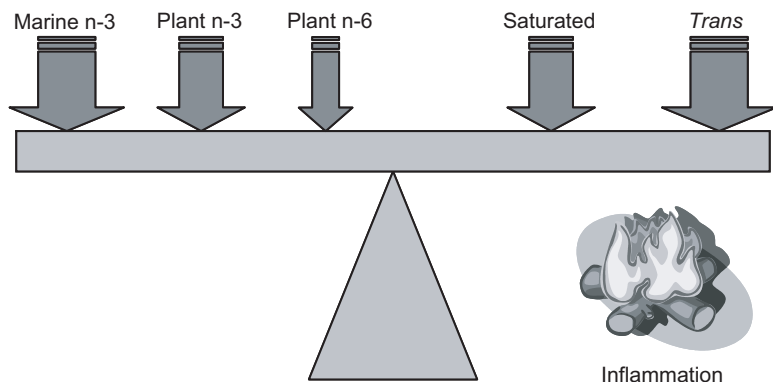


Fig. 18.4 Qualitative depiction of the relative effects of different types of fatty acid on inflammation. Saturated and *trans* fatty acids promote inflammation with *trans* fatty acids probably having a greater impact than saturated fatty acids. Plant-derived n-6 fatty acids and n-3 fatty acids inhibit inflammation; n-3 fatty acids have a greater impact than plant-derived n-6 fatty acids and within the n-3 fatty acid family marine-derived (EPA and DHA) have a greater impact than plant-derived (ALNA).

18.4.2 The hierarchy of effects of fatty acids on inflammation

The findings from studies of fatty acids and inflammation in humans (as reported above) allow a hierarchy of effects to be identified (Fig. 18.4). This is supported by animal feeding experiments, not discussed here, and by established and emerging mechanistic understandings. Amongst the different long-chain fatty acids, *trans* and saturated fatty acids enhance inflammation, with an indication that the effect of *trans* may be greater than that of saturated fatty acids (Baer *et al.*, 2004). Replacing saturated fatty acids with polyunsaturated fatty acids, or specifically with linoleic acid or ALNA, decreases inflammation. High intakes of ALNA decrease inflammation when they replace linoleic acid or when they markedly lower the ratio of linoleic acid to ALNA being consumed. In turn, marine n-3 fatty acids (the combination of EPA+DHA) are more potent than ALNA at reducing inflammation. It is important to note, however, that there are inconsistencies in the findings of observational and interventional studies. These most likely relate to differences in duration of treatment in intervention studies; sample size; inherent characteristics of the populations studied such as age, health status, background diet and genetics; dose of the fatty acid used in intervention studies; whether the fatty acid replaced another or was simply added on top of the diet; and likely many other factors.

18.5 Conclusion

Fatty acids can influence inflammatory cell function and inflammation through a variety of mechanisms, including acting via cell surface and intracellular receptors that control inflammatory cell signalling and gene expression patterns. Some

effects of fatty acids on inflammatory processes involve lipid mediators generated from the fatty acids themselves. Often these fatty acids will be released from cell membrane phospholipids prior to their conversion to the bioactive mediators.

Cells involved in the inflammatory response are typically rich in the n-6 fatty acid ARA which is a precursor to inflammatory eicosanoids. The membrane contents of ARA and of the n-3 fatty acids EPA and DHA can be altered through oral administration of EPA and DHA. EPA also gives rise to eicosanoids, and these often have differing properties from those of the ARA-derived analogues, typically being less potent. EPA and DHA give rise to resolvins, and DHA to protectins, which are anti-inflammatory and inflammation resolving. These relatively recently discovered mediators provide a novel mechanism by which n-3 fatty acids can influence inflammatory processes. Thus marine n-3 fatty acids reduce inflammation and promote its resolution through a number of actions. The plant n-3 fatty acid ALNA can also reduce inflammation, and its effects appear to relate to its conversion to EPA, explaining the lower potency of ALNA compared with marine n-3 fatty acids.

In contrast to the anti-inflammatory actions of n-3 fatty acids, saturated fatty acids seem to enhance inflammation, acting to increase NFκB activation and inflammatory gene expression. Interestingly, in cell culture models marine n-3 fatty acids, especially DHA, can prevent the effect of saturated fatty acids. Human studies demonstrate that *trans* fatty acids are pro-inflammatory, perhaps more so than saturated fatty acids. These considerations establish that *trans* and saturated fatty acids promote greater inflammatory responses and that n-3 fatty acids are anti-inflammatory, with those of marine origin being more potent than the plant-derived ALNA.

Consideration of the impact of n-6 fatty acids is more complex. Since ARA is the precursor to eicosanoids known to have a central role in inflammation and because drugs that target ARA metabolism have some efficacy as anti-inflammatories, the widely held view has been that n-6 fatty acids must, almost by definition, promote inflammation. This view is reinforced by knowledge that marine n-3 fatty acids are anti-inflammatory and that they work, at least in part, by interfering with ARA metabolism. However, the relation of ARA and ARA-derived eicosanoids to inflammation is more complex than first realized, with anti-inflammatory and inflammation resolving effects being identified. This 'middle ground' occupied by n-6 fatty acids is borne out by human studies. Observational and interventional studies suggest that replacing saturated fatty acids with linoleic acid reduces inflammation; thus linoleic acid must be 'less inflammatory' than saturated fatty acids and could even be said to be 'anti-inflammatory' compared with saturated fatty acids. On the other hand, replacing linoleic acid with ALNA decreases inflammation, indicating that linoleic acid is 'less anti-inflammatory' than ALNA and could therefore be considered to be 'pro-inflammatory' relative to ALNA. The difference between linoleic acid and marine n-3 fatty acids will be even stronger. If the aim is to reduce inflammation, then replacing *trans* and saturated fatty acids in the diet with PUFAs would be one strategy. Amongst the PUFAs there may be a place for linoleic acid, but it would be more important to increase intake of ALNA and even more importantly of EPA+DHA.

The multiple mechanisms involved in the interaction of fatty acids with inflammatory cells create a complex picture. Currently these multiple mechanisms of action are not fully integrated, but it seems likely that alterations in cell membrane composition are a key event since such alterations can influence lipid mediator profiles, membrane receptor function and cell signalling processes.

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19

Olive oil and immune function

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Abstract: Animal studies suggest that olive oil modulates immune function *in vivo* and *ex vivo* when fed at high levels. In contrast, consumption of an olive oil-rich diet by humans does not appear to bring about a general suppression of immune cell function, but may modulate levels of cellular and circulating inflammatory biomarkers. The lack of a clear effect of olive oil in humans may be attributable to the higher level of monounsaturated fat used in the animal studies. There is some evidence that the effects of olive oil on immune function in animal studies are due to oleic acid, but there is also growing evidence that the non-lipid fraction, containing polyphenolic components, may influence some aspects of immune function, particularly inflammation.

Key words: olive oil, natural killer cell, lymphocyte, inflammation, Mediterranean diet.

19.1 Introduction

Alteration of the fatty acid composition of the diet can modulate the fatty acid composition of cells of the immune system, and subsequently their function. However, to evaluate the full impact of olive oil on immune function, it is important to consider the effects of both the lipid and non-lipid components. This chapter evaluates the evidence for the effects of olive oil on immune responses in both animals and humans, presents evidence suggesting that both oleic acid and the non-lipid components of olive oil can separately modulate selected immune responses, discusses the use of olive oil-containing lipid emulsions in clinical settings, and reviews evidence for protective effects of olive oil in chronic inflammatory disorders.

19.2 The effects of olive oil on various parameters of immune function

19.2.1 Effects of olive oil on *ex vivo* lymphocyte proliferation

The *in vitro* effects of fatty acids on lymphocyte proliferation have been studied since the early 1970s and have been reviewed in detail elsewhere.¹ These studies investigated the effects of a wide range of fatty acids, including oleic acid, on immune function, but the results are disparate and comparisons between studies are made difficult by the differences in the concentrations of fatty acids used, the cell type studied, the means by which they were presented to cells and the conditions of incubation. The *in vitro* studies therefore remain contradictory, some showing no effect of oleic acid and some showing suppression of lymphocyte proliferation.¹

A number of animal studies have examined the effects of dietary fats on *ex vivo* lymphocyte proliferation. In one such series of dietary studies, the effects of feeding rats a range of high-fat (200 g/kg) diets, each with a characteristic fatty acid composition, or a low-fat (25 g/kg corn oil) diet on lymphocyte fatty acid composition and on a number of lymphocyte functions were compared.²⁻⁴ In these studies, the animals were fed on hydrogenated coconut oil, olive oil, safflower oil, evening primrose oil or fish (menhaden) oil for a period of 10 weeks. The first of these studies reported a significant suppressive effect of olive oil on the *ex vivo* proliferation of mesenteric lymph node lymphocytes in response to the T-cell mitogen, Concanavalin A (Con A), when compared with feeding a low-fat diet or diets rich in hydrogenated coconut oil or safflower oil.² The effect of the olive oil diet was similar in magnitude to that resulting from feeding of fish oil or evening primrose oil² and was also demonstrated in whole blood cultures stimulated with Con A.³ All of the high-fat diets were shown to modulate the fatty acid composition of lymphocytes, resulting in characteristic profiles for each dietary group.⁵

In contrast to these studies, De Pablo *et al.*⁶ demonstrated that olive oil and hydrogenated coconut oil enhanced mitogen-stimulated lymphocyte proliferation compared with sunflower oil (all fed at 150 g/kg) in Balb/c mice. However, it may be important to note that in the study by De Pablo *et al.*,⁶ lymphocytes were cultured in 10% foetal calf serum prior to assessment, whereas Yaqoob *et al.* conducted experiments using autologous serum or whole blood cultures and showed that culturing cells for 48 hours in foetal calf serum, but not autologous serum, reversed the changes in fatty acid composition brought about by dietary lipid manipulation⁵ and masked the effects of dietary lipid manipulation on cell function.² Thus the effects of dietary fats on immune function could potentially be altered by subsequent cell culture conditions.

This issue is also relevant to the study of Berger *et al.*,⁷ which compared the effects of feeding a low-fat (30 g/kg fat), olive oil (100 g/kg), safflower oil (100 g/kg), linseed oil (100 g/kg) or fish plus safflower oil (90+10 g/kg) diet to dams for five months on the proliferation of Con A-stimulated murine spleen lymphocytes of their pups prior to weaning. They reported no effect of dietary manipulation. This could be due to the fact that the fat content of the diets was half that employed by Yaqoob *et al.*, and lower than that used by De Pablo *et al.*, and also to the fact

that, Berger *et al.* fed murine dams on each of the diets and subsequently tested lymphocyte proliferation using cells from the pups before weaning, making it possible that the suckling pups had not been exposed to milk of sufficiently differing fatty acid composition to allow dietary lipid manipulation to occur through this transition. Furthermore, the lymphocytes were cultured in 10% foetal calf serum which, as indicated above, might reverse the changes in fatty acid composition brought about by dietary lipid manipulation and mask any effects of dietary lipid manipulation on cell function.

The studies outlined above used relatively large amounts of a single oil and, as such, they represent rather extreme diets, which are unlikely to be encountered by free-living human beings. Furthermore, the use of such oils inevitably results in variation in the levels of several fatty acids together and not only the one under investigation. A further study therefore investigated the effects of relatively small changes in the levels of commonly consumed fatty acids in a controlled manner in which one fatty acid was exchanged for another, without altering the levels of other fatty acids in the diet.⁸ The nine diets used in this study contained 178 g fat/kg, and differed in their proportions of palmitic, oleic, linoleic and α -linolenic acids whilst maintaining the ratio of *n*-6:*n*-3 polyunsaturated fatty acids (PUFA) at 7. The effect on lymphocyte proliferation of replacing one fatty acid with another appeared to be influenced by the level of other fatty acids in the diet, such that lymphocyte proliferation was decreased with increasing dietary oleic acid levels up to an oleic acid level of 35.6 g/100g fatty acids, but increasing the oleic acid level above this level did not result in any further increase.⁸

One study to date examined the effects of a monounsaturated fat (MUFA)-enriched diet on lymphocyte proliferation in humans.⁹ In this study, middle-aged men were randomly assigned to consume either a control diet (designed to reproduce the current UK diet fatty acid composition) or a diet containing foods enriched with highly-refined olive oil for eight weeks. Foods provided for subjects included the main meal of the day (as a frozen recipe meal), cooking oils and spreads, biscuits and puddings. Subjects on the MUFA diet consumed significantly less saturated fat (% energy) compared with those on the control diet and significantly more MUFA; MUFA contributed 18.4% energy in this group compared with 11.3% in the control group.⁹ Consumption of the MUFA-rich diet did not affect the proliferative response of either whole blood cultures or peripheral blood mononuclear cells (PBMNC) to the T-cell mitogen, Con A.⁹ This observation contrasts with results obtained by the same group using laboratory animals.^{2,3} The lack of a clear effect of MUFA may be attributable to the higher level of monounsaturated fat used in the animal studies, where rats were fed for 10 weeks on diets containing 200 g/kg olive oil (MUFA therefore contributed approximately 30% of total energy intake), whereas in the human study, MUFA contributed approximately 18% of the total energy intake. While it is possible that a higher level of dietary MUFA may have resulted in suppression of proliferation, it is clear that at levels corresponding to Mediterranean intakes¹⁰ and levels which can readily be achieved through consumption of meals which use olive oil as the primary cooking fat, MUFA do not affect lymphocyte proliferation.

19.2.2 Effects of olive oil on *ex vivo* natural killer cell activity

One of the most important mechanisms by which the immune system deals with foreign cells is to damage or destroy them. Typical target cells include malignant cells, normal cells of the host that are infected with viruses or other micro-organisms and normal cells from individuals unrelated to the responding host. Natural killer (NK) cells are a subset of lymphocytes found mainly in blood and the spleen. They are derived from the bone marrow but are neither T cells nor B cells, and they do not undergo thymic maturation. Killing by NK cells is part of natural rather than specific immunity, since it is not induced by a specific antigen and is not restricted by major histocompatibility (MHC) molecules.

Feeding rats for 10 weeks on a diet containing 200 g/kg olive oil resulted in significant suppression of NK cell activity compared with feeding a low-fat diet or diets containing 200 g/kg hydrogenated coconut oil or safflower oil (Fig. 19.1).¹¹ The inhibition of NK activity was greater than that produced by feeding a diet rich in evening primrose oil, but not as great as that resulting from feeding a diet containing 200 g/kg fish oil.¹¹ In the study by De Pablo *et al.*,⁶ an olive oil-rich diet (15% by weight) significantly suppressed NK cell activity compared with hydrogenated coconut oil and, at some time points, sunflower oil, in Balb/c mice. This effect was observed despite the fact that cells were incubated in the presence of foetal calf serum, although in this case for a much shorter duration (four hours) than in the proliferation experiments. Thus, it is possible that there was insufficient

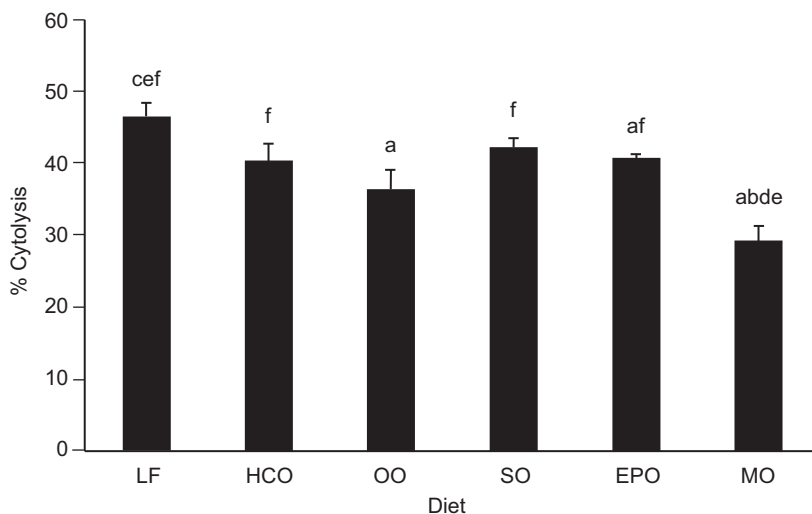


Fig. 19.1 The effect of dietary lipid manipulation on NK activity in freshly prepared rat spleen lymphocytes. Cytolysis of YAC-1 (target) cells by rat spleen lymphocytes (effector cells) was measured by release of ^{51}Cr by preloaded YAC-1 cells at a ratio of 100:1 effector:target cells. Results are expressed as % cytotoxicity. Statistical significance for $P < 0.05$ at least is indicated as follows: ^avs LF, ^bvs HCO, ^cvs OO, ^dvs SO, ^evs EPO, ^fvs MO. LF, low-fat; HCO, hydrogenated coconut oil; OO, olive oil; SO, safflower oil; EPO, evening primrose oil; MO, menhaden oil. Data are taken from reference 11.

time for the foetal calf serum to modify the dietary lipid-induced changes in cell composition and reverse the effects in these experiments. However, Berger *et al.*⁷ showed no effect of an olive oil-rich diet on NK cell activity in mice when compared with a low-fat, safflower oil or linseed oil diet; once again, the lack of effect may be attributable to the amount of fat in the diet and/or the protocol used (dams fed for five months and pups subsequently used prior to weaning).

In a study comparing the effects of nine diets containing 178 g fat/kg, and differing in their proportions of palmitic, oleic, linoleic and α -linolenic acids, on NK activity, there was a significant negative linear relationship between the oleic acid content of the diet and NK cell activity, suggesting that dietary oleic acid suppresses NK cell activity.⁸ Furthermore, there was a negative relationship between the oleic acid: linoleic acid ratio in the diet and NK cell activity and a weak negative relationship ($r = -0.289$; $P = 0.092$) between NK cell activity and the level of oleic acid in spleen lymphocytes.⁸

In healthy, middle-aged men, consumption of a MUFA diet produced a small decrease in NK cell activity at two months, but not one month.⁹ However, this was not statistically significant when compared either with the baseline or with the control group. NK cell activity was unaffected by consumption of the control diet.⁹ As with the effects of olive oil on lymphocyte proliferation, this observation contrasts with some of the animal studies, and may be attributable to the higher level of monounsaturated fat used in the animal studies.

19.2.3 Effects of olive oil on expression of adhesion molecules

Adhesion molecules have pathophysiological as well as physiological roles; for example, adhesion molecules have been implicated in the transendothelial migration of leukocytes into synovial tissue and fluid in rheumatoid arthritis and in leukocyte–endothelium interactions. There has consequently been a great deal of interest in recent years in the potential modulation of the expression and/or functions of adhesion molecules by fatty acids.

In a study by De Caterina *et al.*,¹² human saphenous vein endothelial cells (HSVEC) were preincubated for 24 hours with 10 μ M arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) or oleic acid (OA) prior to six hours of stimulation with tumour necrosis factor (TNF α) and subsequent measurement of the surface expression of vascular cell adhesion molecule-1 (VCAM-1). It was reported that DHA (but not EPA) and OA significantly decreased the expression of VCAM-1 by HSVEC.¹² In a further study, the same group demonstrated that OA inhibited the adhesion of a monocytic cell line to human umbilical vein endothelial cells *in vitro*, but that the effects were less potent than those of PUFA.¹³ In a dietary study comparing the effects of a low-fat diet (25 g/kg) and high-fat diets containing 200 g/kg hydrogenated coconut oil, olive oil, safflower oil, evening primrose oil or fish oil, the level of expression of the adhesion molecules CD2, intercellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen-1 (LFA-1) on rat spleen lymphocytes was

decreased by both olive oil and fish oil.¹⁴ A further study demonstrated that the same diets decreased the adhesion of both freshly-prepared and mitogen-stimulated lymphocytes to macrophage monolayers and the adhesion of mitogen-stimulated lymphocytes to endothelial cells *ex vivo*.¹⁵

A MUFA-rich diet resulted in a significant decrease in the expression of the leucocyte adhesion molecule ICAM-1, on monocytes after two months compared both with baseline values and with those from the control group in a human study.⁹ The expression of ICAM-1 did not change during the consumption of the control diet.⁹ These results suggest that olive oil may have some antiatherogenic and anti-inflammatory properties, which could be useful in the maintenance of health.

Further evidence suggests that a MUFA-rich diet may indeed affect the process of cellular adhesion in humans. In an interesting study by Mata *et al.*¹⁶ healthy men and women living in a religious community were subjected to four consecutive dietary periods (isocaloric) differing in the fat content of saturated fatty acid (SFA), MUFA and *n*-3 and *n*-6 PUFA. It was reported that low density lipoprotein (LDL)-induced monocyte adhesion to endothelial cells was lower during the MUFA period than each of the others and that resistance of LDL to oxidation was greatest during the MUFA period.¹⁶ The authors suggested that the modulation of LDL fatty acid composition was responsible for the differences in adhesion and showed a significant negative correlation between monocyte adhesion to endothelial cells and the OA content of LDL.¹⁶ Expression of adhesion molecules by either cell type was not measured, but it is possible that this too may have played some role in the decreased adhesion during the MUFA period.

A similar study by Tsimikas *et al.*¹⁷ investigated the ability of oxidised LDL from Greek, American and Greek–American subjects to induce monocyte chemotaxis and adhesion to endothelial cells. They also demonstrated a strong negative correlation between the OA content of LDL and both monocyte chemotaxis and adhesion, while there was a strong positive correlation between LDL PUFA content and monocyte adhesion.¹⁷

19.2.4 Effects of olive oil on *in vivo* immune responses

Several *in vivo* studies report immunomodulatory effects of olive oil. Mulrooney and Grimble¹⁸ examined the influence of dietary fatty acids on the inflammatory response to TNF α in rats. This situation mimics the invasion of the body by infective and inflammatory agents, which result in the release of cytokines from cells of the immune system. The purpose of the released cytokines, apart from modulation of the immune system, is to bring about enhanced lipolysis, gluconeogenesis, muscle proteolysis and redistribution of tissue zinc in order to provide substrates for cells of the immune system and amino acids for the synthesis of acute-phase proteins. When weanling rats were fed for eight weeks on diets containing 100 g/kg fat in the form of corn, fish, coconut oils or butter (rich in oleic acid) before an intraperitoneal injection of recombinant human TNF α , the increase in hepatic zinc concentration normally observed in the ensuing response did not occur in animals fed on the fish oil or butter diets¹⁸ and the increase in plasma

caeruloplasmin was smaller in the butter-fed animals than those fed on the other diets.¹⁸ There was also no increase in the rate of protein synthesis in response to TNF α in the livers of animals fed the butter diet, whereas animals fed on the corn oil and coconut oil diets demonstrated the normal increase in protein synthesis associated with the acute phase response.¹⁸

In a subsequent study, it was demonstrated that diets containing 50 or 100 g/kg butter or olive oil completely suppressed the increases in tissue zinc content, liver protein synthesis and serum caeruloplasmin levels in response to subcutaneous *Escherichia coli* endotoxin, when compared with a maize oil diet or standard laboratory chow.¹⁹ In both studies, the butter and olive oil diets decreased the intensity of anorexia induced by TNF α or endotoxin,¹⁹ demonstrating clearly the diminished susceptibility to the lethal effects of both agents in experimental animals.

An alternative experimental model for *in vivo* immune responses is the 'graft versus host' (Gv.H) response, which can be elicited in rodents by injection of allogenic cells into the footpad of a host. The response primarily involves the polyclonal activation and proliferation of host B cells. The 'host versus graft' (Hv.G) response, on the other hand, is a T cell-mediated response, in which cytotoxic T lymphocytes of the host recognise MHC antigens on the injected cells. In both cases, the enlargement of the popliteal lymph nodes (more than 15-fold in the Gv.H response and four-fold in the Hv.G response) is due largely to proliferation of activated host cells within the lymph node, although there is also recruitment of cells from the bloodstream.

Using this assay, Sanderson *et al.*²⁰ demonstrated that feeding rats a diet containing 200 g/kg fish oil suppressed the Gv.H response compared with feeding a low-fat diet or diets containing 200 g/kg coconut oil, safflower oil or evening primrose oil; feeding a diet containing 200 g/kg olive oil had a similar effect, although the response was depressed only compared with the low-fat and evening primrose oil diets (Fig. 19.2).²⁰ The expression of the adhesion molecules LFA-1 and ICAM-1 on lymphocytes from popliteal lymph nodes following a Gv.H response was significantly lower in animals fed the olive oil or fish oil diets compared with those fed the low-fat or coconut oil diets.²⁰ It was speculated that the smaller popliteal lymph node size in animals fed the fish oil or olive oil diets may result from a suppression of both the activation of cells within the node and of the movement of cells from the bloodstream into the nodes.²⁰ Interestingly, while the fish oil diet had a similar suppressive effect on the Hv.G response as that on the Gv.H response, the olive oil diet had no effect on the Hv.G response.²⁰ It appears, therefore, that in this model, olive oil is able to modulate *in vivo* responses involving B cells, but not those involving cytotoxic T lymphocytes.

In rodent models of mammary carcinogenesis, it has been demonstrated that maternal feeding of a diet containing 150 g/kg olive oil to mice before pregnancy resulted in *stimulation* of most areas of the white pulp responsible for the production of T and B lymphocytes in the spleens of the offspring.^{21,22} In the lymph nodes, olive oil increased the areas of the cortical and mantle layers (the main lymphocyte-producing regions), but decreased the area of the medulla, compared

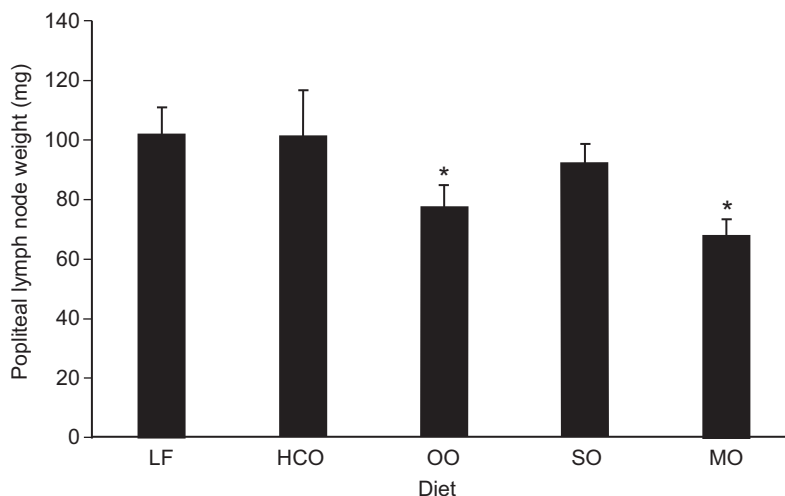


Fig. 19.2 Effects of dietary fatty acids on the graft versus host response. *Denotes a significant difference compared with the low-fat diet. LF, low-fat; HCO, hydrogenated coconut oil; OO, olive oil; SO, safflower oil; MO, menhaden oil. Data are taken from reference 20.

with corn oil.²² However, these experiments were performed in a setting where the offspring were exposed to a carcinogenic agent and therefore reflect the influence of dietary fat on immune responses to tumours, which may be different from that in other settings. Compared with corn oil, the olive oil diet suppressed the appearance of chemically-induced mammary tumours in the offspring.²² This was suggested to be due to enhanced apoptosis of tumour cells, stimulation of the production of lymphocytes in lymphoid tissues and an increase in the infiltration of lymphocytes into the tumour as a result of feeding olive oil.²²

Although there are no published human studies which have set out to examine the effects of olive oil on *in vivo* immune responses, at least one study investigating the effects of fish oil supplements on immunological parameters (including the systemic humoral response to tetanus toxoid) in healthy volunteers has used olive oil as a placebo treatment.²³ The authors claim to demonstrate an immunosuppressive effect of fish oil compared with olive oil, but the protocol is far from satisfactory. Six volunteers were involved in the study, only two of whom received the olive oil treatment and, if the data are scrutinised, it is clear that a larger number of subjects may have produced different results,²³ particularly since measurements of human immune responses are prone to substantial inter-individual variation.

Olive oil and host defence

If olive oil suppresses immune function, it is possible that it will have a detrimental effect on host defence. This was investigated by Wallace *et al.*,²⁴ who examined the influence of a range of dietary fatty acids on macrophage-mediated cytotoxicity towards two tumour cell lines (P815 and L929). Feeding olive oil significantly inhibited the killing of these tumour cells compared with a low-fat diet, but other

high-fat diets, including those containing safflower oil and coconut oil, had similar effects, so it is not clear whether the effect of the olive oil diet was in fact due to amount of fat.²⁴ In the same study, the olive oil diet decreased the *ex vivo* production of TNF α and nitrite by macrophages compared with the low-fat diet but, once again, the effect may have been due to amount of fat.²⁴ Only a fish oil-containing diet appeared to have a specific effect on the responses described above.²⁴

Puertollano *et al.*²⁵ examined the effects of feeding a low-fat diet or high-fat diets containing 200g/kg hydrogenated coconut oil, olive oil or fish oil to Balb/c mice on *in vitro* cellular responses to *Listeria monocytogenes*. Feeding olive oil did not affect spleen lymphocyte proliferation, but it enhanced the cytotoxicity of the pathogen towards splenic cells compared with the low-fat and the hydrogenated coconut oil diets, suggesting a potentially detrimental effect of olive oil.²⁴ However, feeding olive oil did not affect the ability of *L. monocytogenes* to adhere to or invade the cells *in vitro*,²⁵ nor did it affect survival.²⁶

The same group also investigated the effects of hydrogenated coconut oil, sunflower oil and olive oil diets on phagocytic activity in Balb/c mice and demonstrated that the olive oil diet enhanced phagocytic activity and production of interleukin(IL)-1 relative to the other groups.⁶ On the other hand, mice fed an olive oil-rich diet (110 g/kg) during isolation stress exhibited delayed clearance of a fungal infection, although the reasons for this are not clear, and effects on cytokine production in the animals were inconsistent.^{27,28}

19.3 Olive oil-containing emulsions in clinical nutrition

There has been considerable interest in manipulation of the fatty acid composition of lipid emulsions used in nutritional support for critically ill and surgical patients. The basis for the use of emulsions containing lipids other than the traditional soybean oil is that, due to its high content of linoleic acid, soybean oil might promote the generation of AA-derived eicosanoids and exaggerate the inflammatory responses seen in clinical situations involving stress or trauma. Whether this phenomenon does in fact occur *in vivo* and whether it affects clinical outcome is disputed.²⁹ Nevertheless, immunomodulatory interventions aiming to ameliorate the systemic inflammatory response syndrome (SIRS), which is associated with sepsis and multiple organ failure, have focused on the replacement of some of the *n*-6 PUFA with other fatty acids, including MUFA. Proponents of emulsions containing olive oil suggest that it offers an immunologically neutral alternative to soybean oil for use in parenteral nutrition, with the potential benefit of some mild anti-inflammatory effects,^{30–36} although some clinical studies report no differences between olive oil and soybean oil-based emulsions in terms of patient outcome and immunological markers.^{37,38} Granato *et al.*³⁹ compared the effects of an olive oil-based emulsion (ClinOleic™; 80% olive oil and 20% soybean oil) with two soybean oil-based emulsions (Intralipid® and Ivelip®) on a number of immune parameters. Both Intralipid and Ivelip suppressed lymphocyte proliferation and IL-2 production, whereas ClinOleic had no effect, which could be interpreted to

suggest that olive oil-based emulsions offer a neutral alternative to soybean oil-based emulsions with respect to their effects on immune function.³⁹ A similar hypothesis was adopted in a study where rats were subjected to total parenteral nutrition (TPN) in the form of ClinOleic or Ivelip for six days; in this study, there were no significant differences in spleen lymphocyte proliferation, although the response tended to be higher in the ClinOleic group, and the expression of the IL-2 receptor was significantly higher after TPN with ClinOleic.⁴⁰

The neutral nature of olive oil-containing emulsions is also demonstrated in a study by Garnacho-Montero *et al.*,⁴¹ where rats received saline, a soybean oil-based emulsion, a medium-chain triglyceride (MCT)/soybean oil emulsion or an olive oil-containing emulsion prior to challenge with *E. coli*. Both the soybean oil and MCT-containing emulsions diminished bacterial clearance, while the olive oil-containing emulsion did not. However, survival rates were not significantly different between groups.⁴¹ Nevertheless, the effect of olive oil is potentially important, since impairment of phagocytosis and bacterial clearance is an undesirable outcome commonly associated with the use of intravenous lipid emulsions.⁴²

19.4 Olive oil and inflammatory disease

Linou *et al.*^{43,44} compared the relative risk of development of rheumatoid arthritis (RA) in relation to lifelong consumption of olive oil in a Greek population, demonstrating that the risk of developing RA decreased significantly with increased consumption of olive oil, such that individuals in the highest category of olive oil consumption had an odds ratio of 0.39 (95% CI 0.19, 0.82) when compared with those in the lowest category of consumption. This translates as a 2.5 times higher risk of developing RA in individuals with the lowest consumption of olive oil compared to those with the highest consumption. The study, although of great interest, has several limitations, including the fact that it is retrospective and the lifelong assessment of dietary patterns is likely to be very difficult to perform accurately. Nevertheless, it has been noted that the prevalence of RA is greater in Anglo-Saxon populations than in the Mediterranean basin⁴⁵ and that RA seems to be less severe in Mediterranean populations.⁴⁶ While genetics may play a role, only a small proportion of the difference in prevalence has so far been attributed to polymorphisms in human leukocyte antigen (HLA)-DR,⁴⁷ suggesting that a role for diet is still possible.

One further piece of evidence suggests that olive oil may have beneficial effects relating to RA. In a study by Kremer *et al.*⁴⁸ examining the effects of fish oil supplementation on the severity and progression of RA, olive oil was used as a placebo treatment, but clinical evaluations and immunologic tests showed it to have some mild beneficial effects. A total of five out of 45 clinical measures were significantly improved compared with baseline in the olive oil group, eight out of 45 in a low-dose fish oil group and 21 out of 45 in a high-dose fish oil group.⁴⁸ Production of IL-1 by macrophages was decreased in the olive oil group, although not to the same extent as either of the fish oil groups.⁴⁸ The

authors concluded that 'dietary supplementation with olive oil is also associated with certain changes in immune function, which require further investigation.' However, some caution needs to be applied in the interpretation of these results, since it is well known that clinical trials inevitably involve a 'placebo effect'. It could be argued that both the olive oil treatment and the low-dose fish oil treatment represent placebo effects and that the only genuine treatment effect is that of the high-dose fish oil. It is difficult to justify how supplementation of a background diet (likely to contain appreciable quantities of oleic acid) with 9 g/day olive oil could bring about improvement in clinical symptoms in a chronic inflammatory disorder and changes in immune function when substantial alteration of the diet with MUFA failed to affect most parameters of immune function in healthy middle-aged men.⁹

Olive oil has been demonstrated to ameliorate symptoms associated in rats with experimentally-induced ulcerative colitis, a form of inflammatory bowel disease.⁴⁹ Feeding olive oil to rats or mice resulted in significant improvement in both histologically and biochemically assessed disease activity in colon specimens and blood.^{49,50} These observations have not yet been extended to human studies.

Finally, there has been a long-standing interest in the potential protective effects of the Mediterranean diet on cardiovascular disease. Since atherosclerosis is increasingly being recognised to have a chronic inflammatory component, it is possible that at least part of the protective effect of olive oil could be manifested through effects on immune and inflammatory responses which are involved in atherogenesis. In this context, it is interesting to note that dietary olive oil decreased the expression of scavenger receptors in murine macrophages⁵¹ and reduced circulating levels of a number of markers of endothelial activation in humans.⁵² A supplementation study comparing the effects of olive oil and fish oil demonstrated that both treatments lowered plasma fibrinogen levels in women with high baseline fibrinogen, which is a risk factor for cardiovascular disease.⁵³ However, as with the study by Kremer *et al.*⁴⁸ in patients with RA, it is not clear how such a low dose of olive oil (6 g/day) would bring about this effect given the appreciable quantities of MUFA in the background diet.

Most recently, the PREDIMED study compared the effects of a traditional Mediterranean diet, supplemented with either virgin olive oil or with nuts, against a low-fat diet on inflammatory markers in subjects at risk of cardiovascular disease.⁵⁴ After three months, several inflammatory markers (including CD49d, CD40, IL-6 and sICAM-1) were decreased in both Mediterranean diet groups.^{54,55} sVCAM-1 and CRP were decreased by only the Mediterranean plus virgin olive oil diet.⁵⁴ However, the authors of this study note that one of its limitations was that subjects in the low-fat group did not, in fact, alter their habitual diet, and were not actually therefore consuming a low-fat diet. The main differences between the groups were therefore the inclusion of extra virgin olive oil and nuts. A further study by the same group reported effects of the Mediterranean diets on monocyte gene expression.⁵⁶ Thus the nature of the effect of olive oil in inflammatory diseases in general is poorly understood and requires clarification.

19.5 The contribution of non-lipid components to the immunomodulatory effects of olive oil

Since olive oil contains a number of antioxidants, sterols, hydrocarbons and alcohols, it is important to consider whether its effects on immune function, at least in animal studies, are due to oleic acid or to some other component of the oil. This was investigated by Jeffery *et al.*,⁵⁷ who compared the effects of feeding a diet containing high-oleic sunflower oil with a low-fat, olive oil or safflower oil diet on lymphocyte proliferation and NK cell activity in rats. Feeding either the olive oil or the high-oleic sunflower oil diet significantly decreased the proliferation of spleen lymphocytes compared with feeding the low-fat or safflower oil diets; the effects of the olive oil and high-oleic sunflower oil diets were not significantly different from one another.⁵⁷ Similarly, NK cell activity was significantly lower for spleen lymphocytes from rats fed the olive oil or the high-oleic sunflower oil than for those from rats fed the low fat or the safflower oil diets, and the effects of the olive and high-oleic sunflower oils were not significantly different from one another (Fig. 19.3).⁵⁷

This suggests that the effects of the olive oil diet in these animal studies were likely to be due to oleic acid rather than other components of olive oil. Moreover, most animal studies have employed refined olive oil, which would be stripped of most of its polyphenols. However, a human study comparing the effects of extra virgin olive oil with high-oleic sunflower oil on lipid peroxidation and eicosanoid

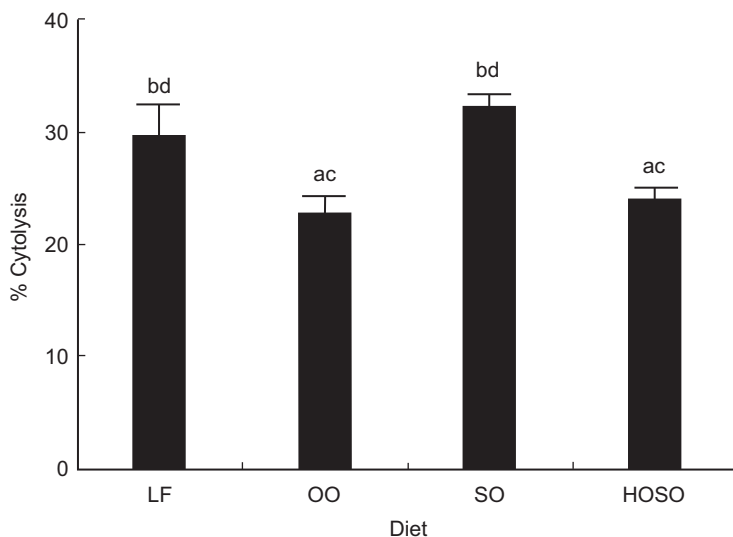


Fig. 19.3 The effect of feeding a high-oleic sunflower oil diet on rat spleen lymphocyte natural killer cell activity. Cytotoxicity of YAC-1 (target) cells by rat spleen lymphocytes (effector cells) was measured by release of ^{51}Cr by preloaded YAC-1 cells at a ratio of 100:1 effector:target cells. Results are expressed as % cytotoxicity. Statistical significance is indicated as: ^avs LF, ^bvs OO, ^cvs SO, ^dvs HOSO. LF, low-fat; OO, olive oil; SO, safflower oil; HOSO, high-oleic sunflower oil. From reference 57, with permission from S. Karger AG, Basel.

production demonstrated that these parameters were higher in individuals consuming high-oleic sunflower oil, which suggests that the non-lipid component of olive oil may exert antioxidant effects *in vivo*.⁵⁸ Furthermore, increasing the amounts of olive oil phenolic extracts administered to human volunteers dose-dependently decreased their excretion of 8-iso-PGF2 α , a marker for oxidative stress.⁵⁹ Evidence that polyphenolic compounds may have direct effects on immune function (either dependent or independent of their antioxidant activities) has been published in the form of studies which have investigated the effects of single olive oil-derived polyphenols on *in vitro* immune responses. Oleuropein and hydroxytyrosol are the principal polyphenolics in olive oil and are potent scavengers of superoxide anion generation and have been demonstrated to inhibit neutrophil respiratory burst^{60,61} and production of IL-1 β (but not other inflammatory cytokines) by human blood cells.⁶² However, oleuropein enhances nitric oxide production by murine macrophages, by increasing both the activity and expression of the inducible form of nitric oxide synthase.⁶⁰ This could be interpreted as a pro-inflammatory action of oleuropein, although it does not appear to be a general feature of flavonoids, since a number of flavonoids inhibit nitric oxide production by macrophages.⁶³ Both oleuropein and hydroxytyrosol have been demonstrated to reduce the adhesion of monocytes to endothelial cells and to reduce the expression of VCAM-1 by endothelial cells *in vitro*.⁶⁴

Since olive oil-derived polyphenolics are essentially hydrophilic, they are abundant in olive oil waste waters (at a concentration about ten times higher than in the oil phase), which are generated during olive oil production and discarded. Visioli *et al.*⁶⁵ demonstrated that olive mill waste water extracts inhibit human LDL oxidation and scavenge superoxide anions and hypochlorous acid at concentrations of 20 ppm. In addition, two of the three extracts used (differing in their degree of refinement) inhibited the production of leukotrienes by human neutrophils and, it was suggested that low molecular weight components, such as hydroxytyrosol, were responsible for the effect.⁶⁵ This is consistent with the observation by De la Puerta *et al.*⁶⁶ that phenolics from virgin olive oil inhibit the leukocyte 5-lipoxygenase. The same group tested the effects of single polyphenolics present in the non-lipid fraction of virgin olive oil in a murine model of inflammation and oedema in the ear. In this model, inflammation was induced by application of AA or a phorbol ester and olive oil phenolics were applied topically to the ear.⁶⁶ The extent of swelling was decreased by between 33 and 45% by oleuropein, hydroxytyrosol, tyrosol and caffeic acid, and each of these compounds inhibited the enzyme myeloperoxidase, suggesting reduced infiltration of neutrophils into the inflamed tissue.⁶⁷

Finally, a rat model of both acute and chronic arthritis compared the effects of a control diet and diets containing high-oleic sunflower oil, virgin olive oil, palm olein, virgin olive oil supplemented with 600 ppm polyphenols and fish oil.⁶⁸ In the model of acute arthritis, the virgin olive oil and polyphenol-supplemented olive oil treatments significantly decreased the inflammation index compared with the control diet, while the other diets had no effect.⁶⁸ In the chronic model, both the polyphenol-supplemented olive oil and fish oil prevented the development of

inflammation to some degree, although the supplemented olive oil had a greater effect.⁶⁸ Thus, these studies demonstrate that the phenolic components of olive oil have some anti-inflammatory activities. It is conceivable, therefore, that oleic acid modulates some aspects of immune function when fed at high levels and that phenolic components of olive oil modulate other functions; in other words, the effects of the lipid and non-lipid fractions of olive oil may be separate and distinct. Whether this is the case in humans *in vivo* remains to be determined.

19.6 Conclusion

Animal studies, depending on the protocol, species and type of measurement, generally support the idea that olive oil is capable of modulating functions of cells of the immune system. The effects appear to be similar to, albeit weaker than, those seen following feeding of diets containing fish oils. There is some evidence that the effects of olive oil on immune function in animal studies are due to oleic acid, but there is also evidence that the polyphenolic components of olive oil have some anti-inflammatory and immunomodulatory properties. Animal models of inflammation, in particular, support this concept.

In contrast, consumption of a MUFA-rich diet by humans does not appear to bring about a general suppression of immune cell functions, but may have some antiatherogenic and anti-inflammatory effects. The lack of a clear effect of MUFA on immune function in humans may be attributable to the higher level of monounsaturated fat used in the animal studies. Human studies investigating the influence of dietary olive oil on immune function have tended to focus on its MUFA content, but potential effects of the non-lipid components of olive oil on immune and inflammatory responses cannot be excluded, particularly since olive oil-derived phenolic compounds are associated with potent antioxidant activity and inhibition of lipoxygenase activity.

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20

The cell- and immune-modulating properties of glutamine

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Abstract: Glutamine is the most abundant free amino acid in the human body. Although glutamine is not an essential amino acid, it is the most important nitrogenous nutrient for diploid cells growing in culture. Glutamine has cell- and immune-modulating properties and affects lymphocyte and monocyte metabolism. Moreover glutamine is one of the precursors of glutathione, stimulates the expression of heat shock proteins and influences gene expression. Glutamine starvation activates adenosine 5'-monophosphate-activated protein kinase (AMPK) and is possibly involved in the metabolism of temporal hibernation. Glutamine is used as a nutritional substrate for enteral and parenteral nutrition (mainly in the form of a stable dipeptide) and has a positive effect on the immune response. Studies on morbidity and mortality are contradictory.

Key words: immunonutrition, amino acids, protein catabolism, lymphocytes, monocytes, glutathione, signalling, ICU, temporal hibernation, adenosine 5'-monophosphate-activated protein kinase (AMPK).

20.1 Introduction

Glutamine is the most abundant free amino acid in the human body and is found in higher quantities and concentrations than any other free amino acid in plasma and in skeletal muscle. Glutamine (GLN) is a non-essential amino acid (AA). Although GLN can be synthesised in all cells from α -ketoglutarate and glutamate via glutamate aminotransferase and glutamine synthetase, studies performed as long ago as 1959 documented that mammalian cells need GLN for an *in vitro* cultivation and that maximum growth of cells was achieved at GLN concentrations of 2 mM, a concentration much higher than that of all other tested amino acids (Eagle, 1959). GLN could not be replaced by α -ketoglutarate (the carbon skeleton of GLN) plus

ammonium chloride or by glutamate. These data indicate that GLN is an essential amino acid for the *in vitro* growth of cells. The lack of essentiality of GLN for the human body is due to the fact that GLN synthesis takes place in skeletal muscle (and partly the lung), using as a substrate other amino acids generated from hydrolysed proteins.

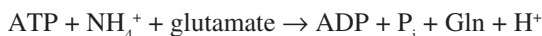
The clinical interest in GLN metabolism was initiated by the facts that GLN is an important substrate for buffering acids in the kidney (Welbourne *et al.*, 1998) and that under severe catabolic conditions an enormous intracellular GLN deficiency in skeletal muscle occurs (Roth, 1982). However, the clinical use of GLN in infusion solutions was limited because GLN is not stable in aqueous solution. GLN became an interesting potential substrate for parenteral nutrition after the development of a method for the synthesis of GLN-containing dipeptides (with either alanine or glycine as corresponding amino acids) which are stable in aqueous solution (Fürst *et al.*, 1989). Subsequent studies revealed that infused GLN-dipeptides are rapidly cleared from the plasma and are therefore well suited for parenteral use (Lochs *et al.*, 1992).

Experimental and clinical results obtained in sepsis revealed that GLN administration could not normalise deficient muscle GLN concentrations (Karner *et al.*, 1989), but was able to regulate cell and immune function. In this respect, it was shown that GLN is involved in the immune response via effects on lymphocytes and monocytes, and that it stimulates heat shock protein (hsp) synthesis, regulates AMP kinase (AMPK) activity in an energy-saving manner, and is important for the regulation of redox potential via the glutathione (GSH) pathway (Roth, 2008). GLN-dependent organs are the kidneys, haemopoietic cells and the intestine. These recent findings indicate that the statement of Sir Hans Krebs that ‘most amino acids have multiple functions, but GLN appears to be the most versatile’ has been proved impressively (Krebs, 1935).

20.2 Chemistry and physiology

GLN and glutamate are 5-carbon amino acids that are structurally similar. At physiologic pH, the carboxyl group of glutamine carries a negative charge, whereas the amino group is protonated, resulting in the molecule carrying a net charge of zero, thus classifying GLN as a neutral amino acid. Glutamate has the amide nitrogen of glutamine replaced by a carboxyl group, conferring to it a net negative charge. This at least partially accounts for the transport of these two amino acids across cell membranes via different transport systems.

GLN is formed from glutamate, which itself is readily convertible to α -ketoglutarate feeding the Krebs cycle. For GLN formation glutamate is enzymatically combined with an ammonium ion (NH_4^+) by the action of glutamine synthetase. Glutamine synthetase is an enzyme which can be found in numerous tissues. It promotes the reaction



In the first step of this reaction, the enzyme-bound high-energy intermediate glutamyl 5-phosphate is formed, an acyl phosphate resulting from the phosphorylation of the 5-carboxyl group of glutamate by ATP. Then in the second step, the glutamyl 5-phosphate combines with an ammonium ion in the active site to form GLN and release phosphate. This energy-dependent reaction is irreversible and tightly regulated. The activity of glutamine synthetase is subject to regulation by over 40 metabolites (Stadtman, 2001). Some of these serve as substrates or allosteric effectors including GLN, glutamate, ammonia, adenosine triphosphate (ATP) and α -ketoglutarate. Thus, glutamine synthetase is regulated by its substrates and products. The rate of GLN formation in skeletal muscle increases in response to enhanced GLN-withdrawal from the plasma by GLN-utilising cells under stress conditions. In contrast, the GLN formation rate decreases when plasma GLN is restored in patients by parenteral GLN administration. This indicates that glutamine synthetase activity in skeletal muscle is regulated to maintain plasma GLN at a defined level.

During starvation and catabolic stress, GLN is mainly produced in skeletal muscle and subsequently delivered to intestinal cells, kidney, and lymphocytes to fulfil the GLN demand of these cells and organs (Cersosimo *et al.*, 1986; Roth, *et al.*, 1988). In the postprandial state, GLN is the only amino acid released continuously from skeletal muscle. This indicates that nature regards a continuous GLN outflow from skeletal muscle for supplying other organs as a necessary requisite of life. Skeletal muscle acts to supply GLN to the rest of the human body during starvation and protein-catabolic situations. Mühlbacher *et al.* (1984) demonstrated that chronic administration of dexamethasone resulted in a four-fold increase in alanine and GLN release from skeletal muscle. Therefore, in stress situations such as post-trauma or during inflammation with an increased production of catecholamines and glucocorticoids, an enhanced release of GLN from skeletal muscle takes place. Under severe catabolic conditions, such as during severe sepsis, the ability of GLN production to meet the demands of other organs results in a decrease in intracellular GLN concentration in muscle and in a relative GLN deficiency state. Under physiological conditions and in the postoperative state, the small intestine is the principal organ of GLN consumption. Windmueller and Spaeth (1974) suggested that the most important cells involved in the intestinal metabolism of this amino acid are the enterocytes, which remove 80–90% of the GLN extracted by the bowel. Forty per cent of GLN nitrogen is converted to ammonia, while 25% appears in alanine. Only a small portion is used for protein synthesis.

20.3 Immune modulation

20.3.1 Importance of glutamine for lymphocytes

Newsholme *et al.* (1985) was the first to investigate the metabolism of glucose, GLN and long-chain fatty acids in lymphocytes. Astonishingly, none of these three substrates was oxidised in great quantities via acetyl-CoA and the classic Krebs

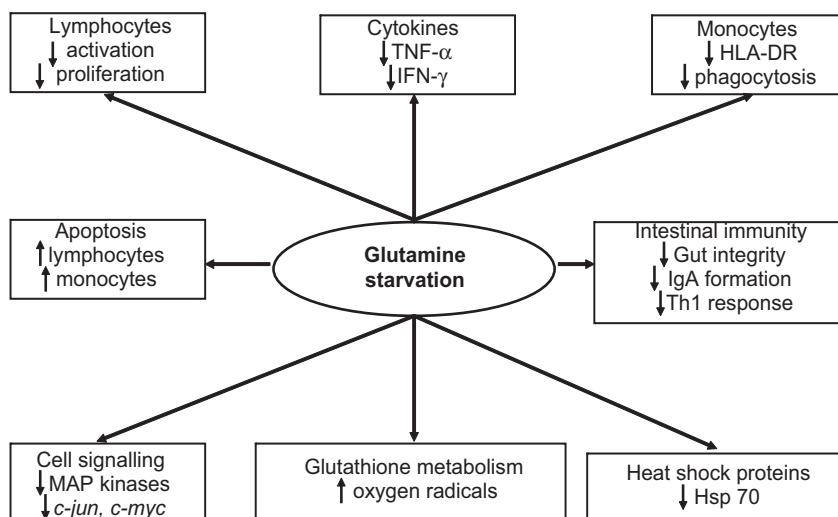


Fig. 20.1 Influence of glutamine starvation on aspects of immune cell function. Glutamine starvation influences several cell systems and pathways leading to a state of immunosuppression.

cycle. Whereas in lymphocytes a high rate of GLN utilisation takes place, glucose is converted almost totally into lactate and the fatty acids are metabolised into ketone bodies. The high rates of glutaminolysis were explained by the need of the cells to respond to an immune challenge at any time. GLN is a known precursor for purine and pyrimidine synthesis and is therefore rapidly needed in proliferating lymphocytes and in activated macrophages for synthesis of cell proteins. Indeed, the proliferation of lymphocytes after activation with mitogenic antibodies directed against the CD3 component of the T-cell receptor (TCR) is highly dependent on the GLN concentration in the cultivation medium (Hörig *et al.*, 1993). A GLN dependence of lymphocytes was also shown for the expression of the cell surface activation markers CD25, CD45RO and CD71, and also for the production of interferon (IFN)- γ and tumour necrosis factor (TNF)- α . Moreover, GLN depletion arrests the cells in the G_0 – G_1 phase, reduces lymphokine-activated killer cell activity and leads to a downregulation of intracellular GSH (Roth, 2007).

However, GLN also seems to stimulate cell growth by influencing cell signalling. In a human glioma cell line it was shown that the oncogen Myc is inducing a transcriptional programme that promotes glutaminolysis and triggers cellular addiction to GLN as a bioenergetic substrate (Wise *et al.*, 2008). Carr *et al.* (2010) recently published that the regulation of GLN uptake by extracellular signal-related kinase (ERK)/MAPK is an important component of T-cell activation, because ERK provides a link to TCR signalling (Fig. 20.1). It was concluded that a better understanding of GLN sensing and use in T cells may reveal novel targets for immune-modulation. In this respect, it should be mentioned that glutamate is also now considered to be an immune-modulator, because glutamate influences

Th17 responses via the specific glutamate receptor mGluR4 on dendritic cells (Hansen and Caspi, 2010; Fallarino *et al.*, 2010).

20.3.2 Glutamine influence on monocytes and differentiation

GLN is an important substrate not only for lymphocytes but also for monocytes/macrophages (Fig. 20.1). Lowering the GLN concentration in culture medium from 2 mmol/L to 200 μ mol/L resulted in a reduced expression of human leukocyte antigen (HLA)-DR by 40% on monocyte-derived macrophages, and decreased tetanus toxoid-induced antigen presentation (Spittler *et al.*, 1995). In addition, low GLN levels downregulated the expression of intracellular adhesion molecule-1 (ICAM-1/CD54), Fc receptor for IgG (Fc γ RI/CD54), and complement receptor type 3 (CR3;CD11b/CD18) and type 4 (CR4;CD11c/CD18). Phagocytosis of IgG-sensitised ox erythrocytes or opsonised *Escherichia coli* correlated with a decreased expression of Fc γ RI and CR3. GLN also has an important impact on the maturation of monocytes along the monocytic pathway (Spittler *et al.*, 1997). Decreasing the GLN concentration causes a reduction in DNA synthesis, increases cell volume and the cytoplasm/nuclear ratio, and enhances the development of vacuoles. Moreover, the percentage of U937 cells phagocytising opsonised *E. coli* or latex particles was enhanced by lowering the GLN concentration. Therefore, reducing GLN concentrations causes differentiation of the U937 cell line along the monocytic pathway.

20.3.3 GLN and intestinal immunity

Sepsis and endotoxaemia markedly influence intestinal GLN metabolism. Souba *et al.* (1990) presented evidence that GLN extraction by the intestine was diminished by 75% in critical illness. This reduced intestinal GLN uptake was accompanied by diminished gut oxygen extraction. On the other hand, endotoxin stimulates lymphocyte glutaminase expression, indicating that intestinal lymphocytes may play a role in increased GLN turnover in endotoxaemia. Kudsk (2003) proved that route and type of nutrition affects mucosal immunity by influencing cell populations within the Peyer's patches (PP) of the small intestine. In subsequent studies, beneficial effects of parenteral/enteral/oral GLN administration on intestinal physiology, including the delivery of energy to the enterocytes, the stimulation of protein synthesis for mucosal regeneration and maintenance of gut integrity, and improvement of intestinal immune reactivity, were all demonstrated (Fig. 20.1). Endotoxaemia in mice significantly reduced the number of lymphocytes and GSH concentrations in the PP. Oral GLN pretreatment could prevent the atrophy of the PP and also the fall of GSH concentration (Manhart *et al.*, 2000a). Parenterally administered GLN increased lymphocyte yield in the PP, in the intraepithelial layer and in the lamina propria (Li *et al.*, 1997), indicating a stimulating effect of GLN on intestinal lymphocytes independent of the route of GLN supply.

In a rat model of experimental colitis induced by 2,4,6-trinitrobenzene sulphonic acid, GLN administration significantly prevented oxidative damage and

lowered the concentrations of TNF and IFN- γ (Kretzmann *et al.*, 2008). In a similar way, the combined administration of GLN and arginine decreased pro-inflammatory cytokine production in the colon of Crohn's patients possibly by causing changes in nuclear factor- κ B (NF κ B) and p38 mitogen-activated protein kinase (MAPK) pathways (Lecleire *et al.*, 2008). Recent data suggest that one of the molecular mechanisms responsible for the gut-protective effect of GLN is the activation of peroxisome proliferator-activated receptor γ (PPAR γ) (Ban and Kozan, 2008).

20.4 Cell modulation

20.4.1 Regulatory capacity of GLN

As already mentioned, GLN has a central role in the amino acid network; it influences energy metabolism, and acts as a precursor for purine and pyrimidine synthesis, thereby influencing lymphocyte and monocyte activity. Moreover, there are several other non-nutritive effects of GLN such as stimulating the formation of hsp, inhibiting cell apoptosis and influencing signalling cascades (Roth, 2008). GLN also influences cell swelling and cell shrinkage which is related to cell and organ anabolism (swelling) and catabolism (shrinkage). In critically ill patients, the volume of muscle cells is decreased, and this decrease is significantly correlated with lowered GLN concentrations in skeletal muscle and also with the total nitrogen loss (Häussinger *et al.*, 1993). On the other hand, when adding GLN to cultured hepatocytes an increased synthesis of DNA, RNA and proteins takes place (Häussinger *et al.*, 1994).

GLN increases the formation of hsp up to 10 000 fold in *Drosophila* Kc cells (Sanders and Kon, 1991) and up to 2.5-fold in the monocyte U937 cell line (Fig. 20.1). The upregulation of hsp 70 in monocytic cells has a protective effect after a hypertonic challenge (Oehler *et al.*, 1998). This cytoprotective effect seems to result from protecting the intracellular ion milieu by influencing ion exchange across the cell membrane. GLN starvation affects the synthesis of stress proteins and especially that of hsp 70. GLN starvation influences the mRNA level of hsp 70 by enhancing mRNA decay (Eliassen *et al.*, 2006a). Monocytic cells cultivated with low amounts of GLN had a drastically shortened half-life of mRNA of hsp 70 (45 minutes vs four hours). Septic patients have a reduced ability to form hsp 70 in lymphocytes (Schroeder *et al.*, 1999). Experimental studies revealed that GLN protects against sepsis and lung injury, possibly by stimulating hsp 70 expression (Singleton and Wischmeyer, 2007) and inhibition of high mobility group box protein-1 expression (Kwon *et al.*, 2010).

20.4.2 GLN and glutathione

GLN via glutamate, cysteine and glycine are the precursor amino acids for synthesis of GSH, which is present within the cell in a reduced (GSH) and an

oxidised form (GSSG). The ratio of GSH:GSSG is the most important regulator of the redox potential which subsequently is critical for various biological events including transcriptional activation of specific genes, modulation of redox-regulated signal transduction and regulation of cell proliferation, apoptosis and inflammation (Rahman, 1999) (Fig. 20.1). In inflammation, GSH is important for the activation of the transcription factor NF κ B and subsequently for the synthesis of certain cytokines and adhesion molecules. GSH synthesis can be altered via the administration of GSH precursors or via the activation of GSH peroxidase and GSH reductase. GSH synthesis is primarily dependent on the supply of cysteine; however, experimental studies revealed that GLN preserves liver GSH after lethal hepatic injury (Hong *et al.*, 1992). The SH-containing tripeptide GSH, is effective in protection of SH-carrying proteins against oxygen radicals and thereby may be especially necessary for dividing cells because they are especially susceptible against reactive oxygen intermediates. In this respect, it should be mentioned that GSH concentration in certain cancer cells is 10–50 times higher than in non-cancer cells, thereby providing an additional protection against oxygen radicals (Godwin *et al.*, 1992).

There is also a significant correlation between GLN supply, intracellular GSH content and rate of lymphocytes proliferation (Roth *et al.*, 2002). The addition of GLN and/or interleukin (IL)-2 increases intracellular gGSH content parallel to an increase of the growth rate of lymphocytes. Dröge *et al.* (1992) reviewed the importance of GSH for lymphocyte function in HIV-infected patients. A depletion of the intracellular GLN pool reduces blast transformation, proliferation and generation of cytotoxic T cells.

In an experimental study, the effect of increased oral supply of either GLN or a mixture of GLN, glycine, arginine and ω -fatty acids on lymphocyte numbers and GSH content in the spleen and in the intestinal PP in endotoxaemic mice was investigated (Manhart *et al.*, 2000a). GLN-fed mice had an increased GSH content in both spleen and PP accompanied by an increased number of lymphocytes. In contrast to GLN supply, the administration of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, significantly reduced the number of lymphocytes in the PP (Manhart, 2000b). These studies clearly indicate that GLN is an effective precursor for GSH synthesis in lymphocytes of PP and the spleen which seems to be associated with an increased number of lymphocytes in the respective organs. These results are confirmed by experiments showing that rats with GSH stores depleted by diethyl maleate had impaired macrophage and T-cell function (Robinson *et al.*, 1993).

20.4.3 GLN apoptosis and autophagy

GSH metabolism is also closely related to apoptotic processes of immune cells (Fig. 20.1). T lymphocytes undergoing apoptosis became dramatically depleted of their reduced GSH content coincident with the onset of chromatin fragmentation (van den Dobbela *et al.*, 1996). In contrast, augmenting intracellular GSH is sufficient to reduce the Fas-triggered increase in apoptosis. An overexpression of

bcl-2, an anti-apoptotic protein, causes redistribution of GSH to the nucleus, thereby altering nuclear redox status and blocking caspase activity as well as other nuclear characteristics of apoptosis.

Apoptosis can be induced by a range of environmental, physical or chemical stresses and is initiated by two principal pathways. The extrinsic pathway is directly activated by the ligation of death receptors such as Fas, whereas the intrinsic pathway (stimulated for example by TNF- α) involves mitochondria. In premonocytic U937 cells the number of apoptotic cells increased only marginally after GLN starvation for four hours. However, GLN starvation renders these cells more susceptible to specific apoptosis triggers (Exner *et al.*, 2002). The number of apoptotic cells was significantly (50–60%) higher in GLN-starved cells when exposed to Fas ligand or heat shock. No effect of GLN starvation was found on UV irradiation-induced apoptosis. These data show that GLN starvation sensitises cells to apoptosis induction, probably by an impaired stress response (Exner *et al.*, 2002).

Studies in myelocytic HL-60 cells revealed an increase in the Fas-mediated apoptosis pathway by GLN starvation (Fumerola *et al.*, 2001). GLN starvation for 12 hours induced a reduction of cell volume by about 40%. This shrinkage promoted a ligand-independent activation of the Fas-mediated apoptosis pathway. Accordingly, cell shrinkage did not induce apoptosis in CD95 receptor-negative lymphoma L1210 cells. Replacement of GLN with surrogate compatible osmolytes counteracted cell volume decrement and protected the CD95-expressing cells from apoptosis.

A similar increase in Fas-mediated apoptosis was observed in GLN-starved HeLa cells (Ko, 2001). Fas-ligation activated ASK1 and Jun nuclear kinase (JNK) in GLN-starved cells but not in normal cells. The authors found a GLN-dependent association of ASK1 with glutaminyl-tRNA synthetase (GLN-RS). ASK1 activity was inhibited by the interaction with GLN-RS. This anti-apoptotic function of GLN-RS was weakened during GLN starvation. Thus, GLN directly interferes with the signal transduction pathway of Fas-mediated apoptosis.

For melanoma cells a recent *in vitro* study hypothesised that glutamine depletion (by administering an inhibitor of glutamate synthetase) plus TRAIL (TNF-related apoptosis-inducing ligand) overcomes the notorious resistance of melanoma to conventional therapy (Qin *et al.*, 2010). However, GLN also interferes with the mitochondrial apoptotic pathway. The exposure of murine primary microglia as well as of the microglial cell line BV-2 to GLN indicates that the hydrolysis of GLN and, accordingly, accumulation of ammonium in mitochondria induce the intrinsic pathway of apoptosis, characterised by mitochondrial dysfunction and activation of caspase-9, which activates caspase-3 (Svoboda and Kerschbaum, 2009).

A recently published animal study revealed that GLN was effective in reducing bacterial translocation, hepatocellular damage, plasma cytokine levels, oxidative tissue damage and apoptosis in jaundice rats (Karatepe *et al.*, 2010). From these studies it can be concluded that the interrelationship of GLN and apoptosis seems to be cell type dependent. The close relationship of GLN metabolism and apoptosis

makes it clear that GLN is also of importance for cancer biology (DeBerardinis and Cheng 2010).

Recently, it was hypothesised that insufficient autophagy in prolonged critical illness may cause inadequate removal of damaged proteins and mitochondria. Such incomplete clearance of cellular damage could explain the lack of recovery from organ failure in prolonged critical illness (Vanhorebeek *et al.*, 2011). Autophagy is a cellular degradation system which controls mitochondrial number and is an adaptive response to the increased metabolic demand upon starvation. Moreover, it was shown that autophagy and glutamine utilisation are reciprocally regulated (Lin *et al.*, 2012). Glutamine deprivation may have a pro-survival role of autophagy in maintaining mitochondrial oxidative phosphorylation and cell growth during metabolic stress. FOXO transcription factors regulate autophagy by modulating intracellular GLN. Conditional activation of FOXO leads to an increased level of GLN production by stimulating GLN-synthetase (van der Voss *et al.*, 2012). This finding reveals that a growth-factor-responsive network can directly modulate autophagy through the regulation of GLN metabolism.

20.5 Influence of glutamine on cell signalling and gene expression

GLN significantly influences cell signalling and gene expression. Rhoads *et al.* (1997, 2009) was the first to show that GLN stimulates intestinal cell proliferation and activates MAPK (Fig. 20.1). This study demonstrated that GLN not only stimulates proliferation by delivering energy but is able to activate ERKs and JNKs. This GLN-dependent activation of the kinases resulted in a four-fold increase in activating-protein 1 (AP-1)-dependent gene transcription. GLN is also required for epidermal growth factor (EGF) signalling through ERKs. Thus, GLN may promote intestinal protein synthesis not only by delivering substrate and energy but also by stimulating transcription of key genes involved in the inflammatory response, cell proliferation, differentiation and metabolic functions (Brasse-Lagnel *et al.*, 2009, 2010). In cell lines, addition of GLN increases expression of the pro-proliferation factors *c-jun* and *c-myc* within a few minutes and promotes cell survival through negative effects on different growth-inhibitory and pro-apoptosis factors. In Ehrlich ascites tumour cells, glutaminase knockout led to enhanced phosphorylation, DNA-binding and transcriptional activity of Sp1.

20.6 Clinical studies with glutamine

GLN administration can be performed orally, enterally or parenterally. Because GLN is not stable in aqueous solution, for parenteral and partly also for enteral administration, stable GLN-containing dipeptides such as L-alanyl-L-glutamine (ALA-GLN) or glycyl-L-glutamine (GLY-GLN) are used (Fürst *et al.*, 1989;

Lochs *et al.*, 1992). Information on the maximum upper limits of GLN supply is available from clinical data and from volunteers receiving GLN (or the corresponding glycyl- or L-alanyl-L-glutamine dipeptides) in dose-finding studies.

20.6.1 Pharmacokinetics of GLN supply

Lowe *et al.* (1990) infused increasing doses of GLN up to 0.570 g/kg/day. This infusion caused a significant increase of plasma GLN concentrations reaching a plateau at approximately 25% above control values. The infusions were well tolerated and neither ammonia nor glutamate, potentially toxic metabolites of GLN, increased significantly. Our group infused increasing daily dosages of GLY-GLN up to 0.570 g/kg GLY-GLN (equivalent to 28 g glutamine for a 70 kg patient) in polytraumatised patients (Weingartmann *et al.*, 1996). This dosage caused a sustained rise in arterial GLN concentrations which remained stable during the four-day study period. No pathological accumulation of free glycine or dipeptide was detected and no side effects occurred. Similarly, the infusion of 0.5 g/kg ALA-GLN (equivalent to 27 g glutamine/70 kg) over four hours in a peripheral vein on three consecutive days was safe and did not cause thrombophlebitis (Berg *et al.*, 2006a). GLN is a precursor of glutamate which may evoke adverse effects by accumulation in the brain; however, even high amounts of ALA-GLN infusions (0.34 g glutamine/kg in 20 hours) to head trauma patients left the cerebral glutamate concentration unaffected (Berg *et al.*, 2006b). The enteral supply of high dosage of glutamine-dipeptide supply (30 g glutamine) in postoperative and critically ill patients and also in cancer patients was well tolerated and metabolically safe (Schroeder *et al.*, 2005). In summary, studies reveal that administration of GLN in a dose up to 30–40 g per 70 kg per day is safe when given for weeks. However, no data are yet available about chronic consumption of GLN to subjects of all age groups.

20.6.2 Parenteral supply of GLN in the ICU

A recently published systematic review of randomised controlled trials of nutrition support with GLN established that parenteral GLN in critical illness is associated with a non-significant reduction in mortality (risk ratio 0.71 (95% CI 0.49, 1.03) and may reduce infection (Avenell, 2009). The authors of this review state that the quality of the studies is poor. There is no evidence to suggest that GLN is harmful in terms of organ failure. Another recently published review was more optimistic in summarising that because exogenous production of GLN may become insufficient during critical illness, the clinical use of GLN should be recommended to critically ill, haematology and oncology patients (Wernerman, 2008).

Interestingly, parenteral GLN supply is advantageous in preventing worsening of insulin sensitivity in multiple-trauma patients (Bakalar *et al.*, 2006). This is of clinical importance because disturbed glucose homeostasis and the resulting hyperglycaemia is a bad prognostic sign.

Two single-centre studies with ICU patients showed significantly reduced six-month mortality with a parenteral nutrition supplemented with 20–25 g GLN per day (Griffiths *et al.*, 1997; Goeters *et al.*, 2002). No survival benefit occurred during the ICU-stay (Griffiths *et al.*, 1997; Goeters *et al.*, 2002). In a study with a low patient number, it was shown that parenteral supply of GLN (0.4 g/kg/day in form of ALA-GLN) reduced infectious morbidity but did not influence mortality (Fuentes-Orozco *et al.*, 2004). The French controlled, randomised, double-blind, multicentre study of total parenteral nutrition (TPN) in critically ill patients showed that ALA-GLN reduced infectious complications and promoted better metabolic tolerance, but did not influence mortality (Dechelotte *et al.*, 2006). Morlion *et al.* (1998) confirmed beneficial effects of GLN-dipeptide-supplemented TPN on lymphocyte recovery and cysteinyl-leukotriene generation. Furukawa *et al.* (2000) demonstrated an enhanced phagocytosis and reactive oxygen intermediate production by neutrophils from postoperative patients *in vitro* when TPN was supplemented with GLN. Exner *et al.* (2003) proved that a perioperative infusion of GLY-GLN caused an accelerated restoration of the lipopolysaccharide-stimulated TNF response after surgery-induced immunosuppression. Cetinbas *et al.* (2010) presented data that GLN-supplemented TPN (0.4 g/kg/day) in patients with systemic inflammatory response syndrome (SIRS) significantly decreases leukocyte and natural killer cell count, parameters representative for an inflammation. However, no significant alterations were found in numbers of B-lymphocytes and immunoglobulin concentrations.

Wernerman (2011) presented evidence that GLN administration (0.283 g GLN/kg/day) in form of the dipeptide ALA-GLN lowered ICU mortality but not mortality at six months. No differences were seen for the SOFA scores. In a randomised trial Andrews *et al.* (2011) determined whether inclusion of GLN, selenium or both in a standard isonitrogenous, isocaloric preparation of parenteral nutrition influenced new infections and mortality among critically ill patients. Parenteral GLN (20.2 g/day), or selenium (500 µg/day), or both given to ICU patients with gastrointestinal failure was not effective in preventing new infection or in reducing mortality. Only patients receiving selenium for less than five days showed a reduction in new infections. Ziegler *et al.* (2012) headed a sophisticated randomised, double-blind, multicentre trial performed in the USA supplementing surgical ICU patients ALA-GLN (0.5 g/kg/day) for an average infusion time of 12–13 days. This is the first study measuring the blood GLN at entry which was in both groups (treatment and control) subnormal (403 vs 437 µM). During GLN infusion, this basic GLN concentration was increased to 33–38%. In spite of these increased GLN concentrations, there were no differences between groups in 28-day hospital (16 vs 15%) or 6-month (30 vs 31%) mortality rates, new total or site-specific hospital infections, ventilator-free days, serial SOFA scores or ICU or hospital length of stay. Similarly, a study giving ALA-GLN (0.6 g/kg/day) to infants with gastrointestinal disease did not reduce the incidence of sepsis nor reduce the time until full enteral feeding was achieved (Ong *et al.*, 2012). These four last studies confirm the results published in the

systematic review by von Avenell (2009) showing no positive influence of parenteral GLN supply on mortality of ICU patients.

20.6.3 Enteral and oral supply of GLN

As already mentioned above, high amounts of enterally supplied GLN did not have any harmful effects on trauma patients (Schroeder *et al.*, 2005). In trauma patients enteral GLN, started 48 hours after the trauma, significantly reduced the incidence of pneumonia, bacteraemia and severe sepsis (Houdijk, *et al.*, 1998). This beneficial effect on the morbidity state could not be reproduced in a more heterogeneous group of ICU patients which were capable of tolerating enteral feeding (Jones *et al.*, 1999). In this study however, the overall post-intervention hospital cost was significantly reduced in enteral GLN recipients.

Long *et al.* (1995) investigated the impact of GLN-supplemented enteral nutrition on whole body protein kinetics and glucose metabolism in trauma patients. They compared an enteral formula providing a mean intake of 0.35 g GLN/kg body weight per day with nutrition providing only 0.05 g GLN. They could not find a difference in nitrogen balance, or whole body protein turnover, synthesis and breakdown between the two groups. They concluded that GLN-enriched formulas do not provide additional nutritional advantage as compared with standard enteral formulas. However, in this study no parameters of inflammation or infection were monitored and therefore the correct conclusion would have been that enteral GLN does not influence overall protein metabolism of trauma patients.

The administration of GLN to cancer patients is under discussion because oncologists often refuse to supply GLN to a tumour-bearing host, since several *in vitro* experiments revealed that cancer cells preferably metabolise GLN. However, GLN can be of advantage in long-term periods of cancer cachexia and may support immunological and organ function. Most of the published clinical studies in oncology have tried to use GLN as a substrate for preventing mucositis/stomatitis in patients receiving chemotherapy. An oral GLN dosage of 7.5 g/day for three weeks administered for preventing mucositis/stomatitis of breast cancer patients receiving anthracycline-based chemotherapy did not cause any side effects and reduced the incidence of clinically significant oral mucositis (Peterson *et al.*, 2007).

Patients with liver diseases seem to be more sensitive to GLN administration. In cirrhotic patients (pre- and post-liver transplantation) the administration of 10 g glutamine significantly increased venous blood ammonia and impaired reaction time following oral GLN challenge (Ortiz *et al.*, 2004). We compared enteral and parenteral GLN supply in patients with transjugular intrahepatic portosystemic shunt and liver cirrhosis and measured mesenteric venous-arterial concentration differences of ammonia and GLN (Plauth *et al.*, 2000). Our data demonstrated that enteral GLN supply resulted in a higher portal ammonia load and a higher degree of systemic hyperammonaemia. Therefore, oral/enteral GLN administration can significantly affect ammonia metabolism in patients with an impaired liver function and therefore has an influence on hepatic coma.

20.7 Future trends

As stated above, data concerning the effects of GLN supply to ICU patients are contradictory. *In vitro* studies have demonstrated that GLN concentrations lower than 300 $\mu\text{mol/L}$ (normal plasma GLN concentrations are approximately 600 $\mu\text{mol/L}$) significantly reduce the functioning of immune cells (Newsholme *et al.*, 1985; Spittler *et al.*, 1995; Oehler *et al.*, 2002; Eliassen *et al.*, 2006a,b). Lowered GLN concentrations affect lymphocyte proliferation, apoptosis, monocyte function and hsp synthesis. These experimental studies suggest that subnormal GLN concentrations might be harmful for humans. In accordance with this idea, plasma GLN depletion is an independent factor determining outcome in critically ill patients (Oudemans-van Straaten *et al.*, 2001). Consequently, in the case of lowered plasma GLN concentrations, an exogenous GLN supply seems to be necessary to support the cellular defence mechanisms (Tjader *et al.*, 2007). From these clinical and experimental data it seems to be clear that clinical studies investigating the effect of GLN supply should be stratified according to the plasma GLN concentrations of the patients.

Although numerous experimental and clinical studies have been carried out attempting to clarify the reason for the intracellular GLN deficiency in skeletal muscle, there is no satisfactory answer. In a recent publication it was hypothesised that reduced GLN concentrations cause a lowering of the intracellular ATP (and an enhanced AMP) level and subsequently a stimulation of AMPK resulting in energy conservation within the cell by shutting down synthetic processes (Roth and Oehler, 2010). This hypothesis fits excellently to an interesting theory stating that in the septic state a 'suspended hibernation' takes place which is analogous to aestivation and hibernation (Singer, 2007; Rudiger *et al.*, 2008). In this case, the cell may focus its use of ATP on processes essential for cell survival, such as maintenance of its membrane potential and autophagy. This hypothesis is strengthened by the observation that, despite organ dysfunction, no extensive cell death in the affected organs can be detected in post mortem studies. Although this is a new concept for multi-organ dysfunction in sepsis, it is a well-described protective strategy in a variety of animals as an adaptive response to heat, cold, drought or starvation. In humans, it is a recognised phenomenon in cardiomyocytes during ischaemic heart disease and persisting hypoperfusion (Heusch, 2005).

Ischaemic heart disease is associated with hibernation-like upregulation of a protective gene programme which helps to reduce myocardial contractility and energy expenditure. Chronic hibernation of the myocardium leads to sustained morphological alterations in the cardiomyocyte, such as loss of myofilaments (myosin, thin filament complex, titin), loss of sarcoplasmic reticulum, an increased number of small, doughnut-like mitochondria, disorganisation of the cytoskeleton (desmin, tubulin, vinculin) and sequestration of cellular particles in the extracellular space. These morphological features of chronic hibernation are reminiscent of skeletal muscle alterations found in the septic state. Indeed, from the available data it seems that GLN metabolism is of particular interest in hibernation (Fig. 20.2). A recent nuclear magnetic resonance (NMR)-based metabolomics analysis in the

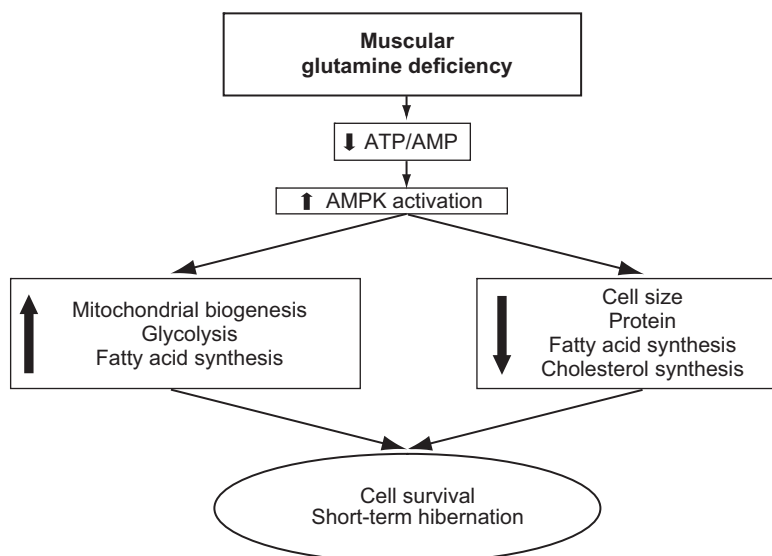


Fig. 20.2 Glutamine deficiency leads to a state of cellular hibernation. Glutamine deficiency reduces ATP levels thereby activating AMP-kinase (AMPK). AMPK activation results in energy conservation within the cell by shutting down synthetic processes. The resulting state of suspended hibernation is possibly necessary in severe diseases such as sepsis for protection of important processes. Therefore, glutamine deficiency in sepsis may be a necessary step for switching cell metabolism into a protected status.

squirrel *Spermophilus tridecemlineatus* revealed that temporal hibernation is associated with alterations in the liver metabolism of the amino acids GLN and alanine (Serkova *et al.*, 2007). A neurochemical profile showed that the concentration of GLN is decreased to a greater extent (–54%) than that of any other amino acid (Henry *et al.*, 2007). These studies show that glutamine deficiency is a common feature in temporary hibernation. During hibernation, skeletal muscle mass is reduced by 14–65% depending on the muscle type and species (Cavey *et al.*, 2003). In addition, muscle strength is reduced by 23%. Therefore, hibernating mammals have been proposed to ‘provide a natural model system to study mechanisms that increase the tolerance of skeletal muscle to atrophy’.

Earlier studies on the impact of GLN starvation on responses of monocytic cells fit this hypothesis (Eliassen *et al.*, 2006a,b). Monocytic cells develop an adaptive cellular mechanism to GLN starvation. When culturing monocytic U937 cells at 0.2 mM GLN for up to four days, there is a decrease of ATP (78% of control), intracellular free GLN (13%), hsp70 (74%) and the cellular proliferation rate (79%). Moreover, in GLN-starved cells, the metabolic sensor AMPK was rapidly increased and only regained normal levels at normal GLN concentration. It is known that AMPK activation is essential for energy-saving strategies suppressing ATP-expensive processes such as synthesis of protein, cholesterol and fatty acid, and stimulating processes responsible for the maintenance of cell integrity such as autophagy. In summary, we hypothesise that GLN deficiencies contribute to

AMPK activation and thereby stimulate energy-conserving processes leading to a hibernation-like state. However, there are several open questions regarding this hypothesis which have to be clarified in future studies.

20.8 Conclusion

GLN is an essential substrate for proliferating cells. GLN metabolism is closely related to the stress-response of the human body and initiates several protective effects against stress and inflammation. Experimental research in cell culture and animal models proved the importance of GLN supply for immunity and inflammation. However, findings from clinical studies with GLN supply to inflamed or infected patients are inconsistent. Some studies show that administration of GLN to septic patients induces an improvement in mortality. On the other side, several studies do not show a beneficial effect of GLN on mortality. This discrepancy may result from the fact that GLN administration may only be useful in patients having lowered GLN pools. Now we have the first study where GLN was indeed infused to ICU patients with lowered blood GLN concentrations (Ziegler *et al.*, 2012). In these patients GLN supply increased blood GLN without having any clinical benefit for the patients. So we have to rethink why glutamine starvation reduces immune reactivity but GLN administration does not seem to be successful in improvement clinical outcome. We also have to consider whether administration of an enormous amount of alanine, which is in an equimolar concentration, to GLN when supplying the stable dipeptide L-ALA-GLN may have a negative influence on the outcome. Alanine is a powerful gluconeogenetic substrate and may even increase hyperglycaemia which is harmful for ICU patients. Another explanation for the unsuccessful supply of GLN in ICU patients may be due to the fact that GLN deprivation stimulates autophagy which is an important process for regulating protein and mitochondria degradation in stress situations (Vanhorrebeek *et al.*, 2011; Lin *et al.*, 2012). A GLN supply may disturb the GLN-autophagy network (van der Vos, 2012) and alter the energy metabolism.

20.9 Sources of further information

There are numerous reviews about GLN and inflammation/immunity which give a deeper insight into the relation of GLN metabolism and inflammation/immunity than given here (Roth 2007, 2008; Avenell, 2009; Brasse-Lagnel *et al.*, 2009; DeBerardinis and Cheng, 2010; Soeters and Green, 2012; Kim and Wischmeyer, 2013).

Tumour incidence seems to be closely related to inflammation and therefore GLN is becoming an interesting substance for cancer research. GLN is an important source of energy for neoplastic tissue. A recent review concentrates on the expression of glutaminase in different neoplastic tissues (Szeliga and Obara-Michlewska, 2009). GLN administration to cancer patients is still under debate,

because GLN can have protective roles during chemotherapy and radiotherapy, but is also a stimulating substrate for proliferating cells (Belabel, 2009). The MYC oncogene, which is frequently deregulated in human cancers, encodes a master transcription factor that integrates cell proliferation with metabolism through its regulation of thousands of genes including microRNAs. In this respect, MYC also regulates glutaminase expression and thereby GLN metabolism and has an impact on cellular energy metabolism which leads to a rethinking of the Warburg effect as a possible target in cancer therapy (Dang *et al.*, 2009, 2010). Skeletal muscle seems to maintain a system of defence against inflammation through hsp and production of myokines. This review shows the relationship between muscle in defence and GLN production of skeletal muscle (Lightfoot *et al.*, 2009). In summary, recent GLN research arouses interest not only in clinical nutrition but also in immunology and cancer research.

20.10 References

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21

Arginine and immune function

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Abstract: L-Arginine (Arg) was traditionally classified as a nutritionally non-essential amino acid for infant and adult humans; however, it is now known that besides serving as a building block for protein, it plays an essential role in the functions of the immune system. Arg is a physiological substrate for the synthesis of nitric oxide (NO), which is a key mediator of immune responses. Additionally, emerging evidence shows that Arg exerts a regulatory role in signal transduction pathways involving mammalian (or mechanistic) targets of rapamycin and specific mitogen-activated protein kinases in immunocytes, thereby affecting immunity. Because of high requirements for Arg in multiple metabolic pathways, dietary Arg supplementation may be a necessary strategy to maintain Arg homeostasis for good health and body functions under many physiological and pathological conditions. Much experimental and clinical data support the notion that Arg is an essential nutrient for both innate and adaptive immune systems in humans and other animals. An important outcome of this research is the availability of Arg-supplemented foods for humans and feed-grade Arg for livestock species. Future studies are needed to address the safety issue of chronic Arg supplementation to mammals so as to fully realize the health benefits of Arg as a functional amino acid.

Key words: amino acids, immune systems, metabolism, nutrition, supplementation.

21.1 Introduction

L-Arginine (Arg) is a basic amino acid present in physiological fluids. Arg has many unique metabolic pathways in mammals because of its positively charged guanidino group on the side chain (Blachier *et al.*, 2011). Based on whole-body nitrogen balance, Arg was traditionally classified as a nutritionally non-essential amino acid for infants and adult humans (Flynn *et al.*, 2002). However, it is now known that besides serving as a building block for protein, Arg plays an essential

role in the functions of multiple systems (including the immune system) and that supplementing Arg to the regular diet is beneficial for improving health and enhancing resistance to infectious disease (Wu *et al.*, 2009). Nitric oxide (NO), which is synthesized from Arg by NO synthase in virtually all cell types, regulates both innate and adaptive immune responses in humans and other animals (Li *et al.*, 2007). Additionally, emerging evidence shows that Arg itself participates in signal transduction pathways in immunocytes by activating mammalian (or mechanistic) target of rapamycin (mTOR) (Salmond and Zamoyska, 2010; Bazer *et al.*, 2012) and specific mitogen-activated protein kinases (MAPK) (Mieulet *et al.*, 2010). Thus, Arg profoundly influences the production of cytokines, the proliferation of lymphocytes and the generation of specific antibodies in response to immunological challenges. The major objective of this chapter is to highlight recent advances in this area of interdisciplinary research involving both protein nutrition and immunology.

21.2 Sources of arginine in the circulation

21.2.1 Dietary source of L-arginine (Arg)

All plant and animal proteins contain Arg (Li *et al.*, 2011). The content of arginine is relatively high in some foods (e.g., seafood, watermelon juice, nuts, seeds, algae, meats, rice protein concentrate and soy protein isolate), but relatively low in the milk of most mammals (including cows, humans and pigs) (Wu *et al.*, 2009). Gelatin is particularly rich in Arg, accounting for approximately 10% of protein (Li *et al.*, 2011). Results of the third National Health and Nutrition Examination Survey indicate that mean Arg intake for the US adult population is 4.4 g/day, with 25, 20 and 10% of people consuming <2.6 (suboptimal), 5–7.5, and >7.5 g/day, respectively (King *et al.*, 2008). While it was traditionally considered that Arg was synthesized adequately in adult humans and was not needed in diets, it is now known that a lack of Arg in the diet severely impairs the reproductive functions of both men and women (Wu *et al.*, 2009) as well as cardiovascular function (Wu and Meininger, 2009), wound healing, immune responses (Li *et al.*, 2007), development of brown adipose tissue (Wu *et al.*, 2012), and energy metabolism (Dai *et al.*, 2013) in mammals, including humans. Notably, home-bound elderly adults (Dasgupta *et al.*, 2005) and preterm infants (who represent 10–12% of newborns) are at great risk for Arg deficiency (Becker *et al.*, 2000) because of low intake and inadequate endogenous synthesis. In clinics, hypoargininemia is manifested as hyperammonemia and multi-organ dysfunction (Wu *et al.*, 2004). A sufficient supply of Arg from the diet is essential for optimal immune function in both humans and other animals.

Dietary proteins are hydrolyzed by proteases in the luminal fluids of the stomach (i.e. pepsins A, B and C and rennin; optimal pH 2 to 3) and of the small intestine (i.e. trypsin, chymotrypsin, elastase, carboxypeptidases A and B, and aminopeptidase; optimal pH 6 to 7.5) to form Arg-containing dipeptides, tripeptides and free Arg (Fig. 21.1). Arg is highly stable in the lumen of the gastrointestinal

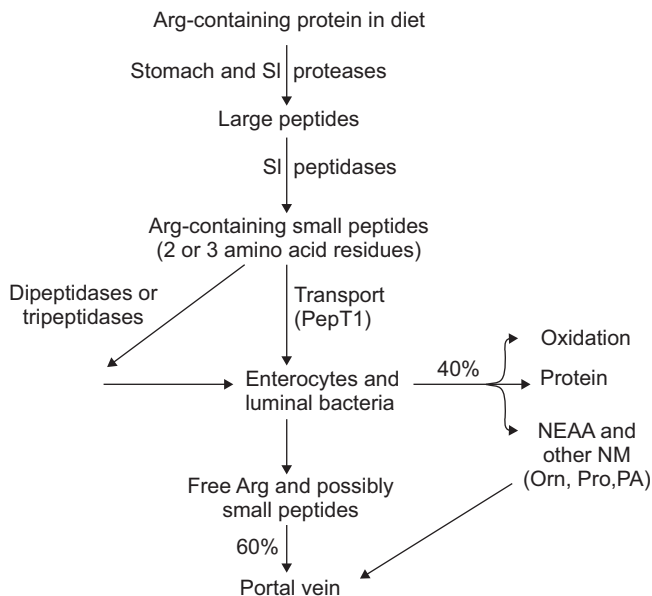


Fig. 21.1 Digestion of dietary protein-bound arginine (Arg) in the gastrointestinal tract of humans and other animals. Proteases and peptidases in the lumen of the small intestine hydrolyze proteins and large peptides, respectively, to eventually form Arg-containing tripeptides and dipeptides. A proportion of these dipeptides are hydrolyzed by peptidases to yield free Arg. The Arg-containing dipeptides or tripeptides in the lumen of the small intestine can be directly transported into the enterocytes through their apical membrane by H^+ gradient-driven peptide transporter 1 (PepT1). This peptide transporter does not transport free Arg or peptides containing four or more amino acid residues. Arg-containing small peptides are rapidly hydrolysed by intracellular peptidases to form free Arg in enterocytes and bacteria. A small proportion of Arg-peptides may exit the enterocytes via their basolateral membrane into the bloodstream, but the identity of basolateral peptide transporters remains unknown. Free Arg in the lumen of the small intestine is taken up by enterocytes primarily via the Na^+ -independent transporters (CAT1, 2 and 3). Approximately 40% of luminal Arg is catabolized by the small intestine, with the responsible cell types including enterocytes, other mucosal cells, and bacteria. CAT, cationic amino acid transporters; NEAA, nutritionally essential AA; NM, nitrogenous metabolites; Orn, ornithine; PA, polyamines; PepT1, H^+ gradient-driven peptide transporter 1; Pro, proline; SI, small intestine.

tract. The alkaline medium in the lumen of the small intestine results from bile salts, pancreatic juice and duodenal secretions. Mucosa-derived peptidases (or prolidases in the case of peptides containing both Arg and proline) can hydrolyze the luminal small peptides to yield free Arg (Wu, 2013). Additionally, Arg-containing dipeptides or tripeptides in the lumen of the small intestine can be directly transported into the absorptive epithelial cells (enterocytes) through their apical membrane by H^+ gradient-driven peptide transporter 1 (PepT1) (Daniel, 2004). Of particular interest, the transport of di- and tripeptides from the lumen into the enterocytes is associated with an influx of both Na^+ and water. Neither free Arg

nor peptides containing four or more amino acid residues are accepted as substrates for PepT1. The Arg-containing small peptides are hydrolyzed rapidly by intracellular peptidases to form free Arg. It is possible that a small proportion of these peptides exit the enterocytes via their basolateral membrane into the bloodstream. Some published studies support the view that a peptide transporter is expressed in the basolateral membrane of the enterocytes for the movement of small peptides from inside the cell into the portal circulation (Gilbert *et al.*, 2008). However, the identity of basolateral peptide transporters remains elusive.

Free Arg in the lumen of the small intestine is taken up by enterocytes primarily via the Na⁺-independent cationic amino acid transporters (CAT1, 2 and 3; also known as system y⁺), which are encoded by SLC7A1, SLC7A2 and SLC7A3 genes, respectively (Deves and Boyd, 1998). To a much lesser extent, Na⁺-dependent transporters (e.g., B⁰⁺, y⁺L and N) and Na⁺-independent transporter b⁰⁺ absorb Arg in the lumen of the small intestine into the enterocytes. In postweaning mammals, these cells contain a high activity of arginase to hydrolyze Arg into urea plus ornithine, which is further metabolized to form proline and polyamines (putrescine, spermidine and spermine) (Wu *et al.*, 1996). The jejunum is the major site for the absorption of dietary Arg, followed by the ileum and duodenum. There is evidence that Arg dipeptides and supplemental (i.e., free) Arg are metabolized similarly in porcine enterocytes (G. Wu, unpublished data).

Not all the free Arg in the lumen of the small intestine is available for utilization by extra-intestinal tissues. The lumen of the small intestine harbors a large number of bacteria that actively degrade dietary amino acids, including Arg (Dai *et al.*, 2010, 2011). The Arg-containing small peptides and free Arg can also be taken up by luminal bacteria via specific transporters (Dai *et al.*, 2011). In bacteria, Arg-containing dipeptides or tripeptides are rapidly hydrolyzed into free Arg, which is utilized via multiple metabolic pathways (Dai *et al.*, 2010). As noted above, the enterocytes of postweaning mammals extensively degrade Arg (Wu *et al.*, 1996). Thus, 40% of dietary Arg is degraded by the small intestine in the first-pass metabolism and substantial amounts of orally administered Arg do not enter the systemic circulation in adults (e.g., humans, pigs and rats) (Castillo *et al.*, 1993; Wu *et al.*, 2007a). Because the transport system y⁺ (a high-affinity, Na⁺-independent transporter of basic amino acids) is virtually absent from hepatocytes, 90% of the Arg delivered to liver is not taken up by this organ (Wu and Morris, 1998). Thus, assuming the digestibility of protein-bound Arg to be 90%, only 50% of the dietary Arg enters the systemic circulation (Jobgen *et al.*, 2006). In contrast, there is little arginase activity in enterocytes of the neonatal small intestine (Rhoads and Wu, 2009) and nearly all of the absorbed dietary Arg that is not utilized locally for protein synthesis can enter the portal vein of suckling infants (Wu *et al.*, 2009). Results from studies of neonatal pigs indicate that only 7–10% of physiological levels of arginine in the portal vein are taken up by the liver (Wu *et al.*, 2007b).

21.2.2 Endogenous synthesis of arginine

Arg is formed as an intermediate of the hepatic urea cycle in the mammalian liver.

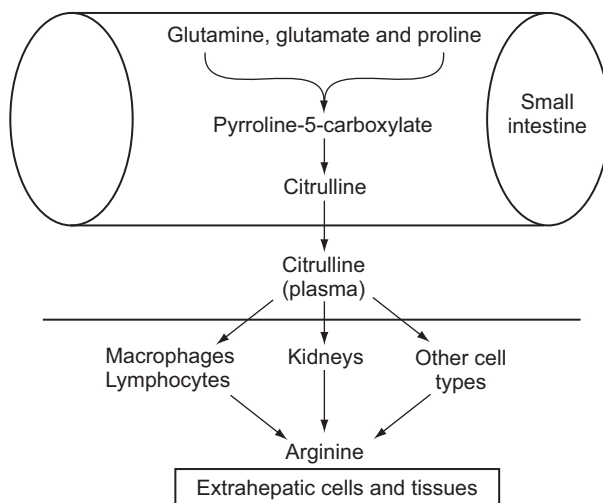


Fig. 21.2 Arginine synthesis in most mammals (including humans) via the intestinal–renal axis. The absorptive epithelial cells (enterocytes) of the small intestine synthesize citrulline from glutamine/glutamate and proline via pyrroline-5-carboxylate as the common intermediate. Citrulline is used for arginine synthesis in both the enterocytes and extraintestinal organs (primarily the kidneys) in neonates. In postweaning mammals and adults, the conversion of citrulline into arginine occurs mainly in the kidneys.

However, there is no net synthesis of Arg via this metabolic cycle because the liver contains an exceedingly high arginase activity to rapidly hydrolyze Arg into urea plus ornithine (Wu and Morris, 1998). Indeed, Arg concentrations in hepatocytes are very low (0.03–0.1 mM), compared with 0.5–10 mM for other amino acids (Li *et al.*, 2009a,b). It is now known that the endogenous synthesis of Arg in most mammals (including humans, pigs and rats) involves the intestinal–renal axis (Wu and Morris, 1998). Namely, citrulline is synthesized from glutamine, glutamate and proline in the mitochondria of enterocytes, released from the small intestine and taken up by extrahepatic cells and tissues (including cells of the immune system and kidneys) for Arg production (Fig. 21.2). In neonates, most of the citrulline synthesized in enterocytes is converted locally into Arg (Wu and Knabe, 1995). Interestingly, the uptake of citrulline by the liver is negligible and this organ is not active in extracting Arg from the circulation (Wu *et al.*, 2007b). Therefore, nearly 100 and 90% of the gut-derived citrulline and Arg, respectively, bypass the liver in pigs (Wu *et al.*, 2007b). Similar patterns of citrulline and Arg metabolism have recently been reported for humans (van de Poll *et al.*, 2007; Ligthart-Melis *et al.*, 2008). Based on physiological needs (e.g., spermatogenesis and placental growth, including vascular growth) beyond protein synthesis, endogenous synthesis of Arg is inadequate in adult males and pregnant females (Wu *et al.*, 2009).

Pyrroline-5-carboxylate (P5C) synthase, proline oxidase and N-acetylglutamate (NAG) synthase are the three key regulatory enzymes of intestinal citrulline synthesis (Wu and Morris, 1998). The enterocyte is the only mammalian cell type

that expresses all three of these enzymes, indicating an essential role for the gut in whole-body homeostasis of citrulline and Arg (Flynn and Wu, 1996). NAG is an allosteric activator of both P5C synthase and carbamoylphosphate synthase-I, thereby regulating citrulline production by enterocytes (Wu *et al.*, 2004). Thus, the integrity and function of enterocytes plays a crucial role in defending the host against infectious disease. Results from recent studies with growing pigs indicate that supplementing Arg (up to 1.1% on the basis of dry matter) to the diet did not affect the synthesis of citrulline and arginine from glutamine/glutamate and proline by enterocytes (G. Wu, unpublished data). Therefore, Arg supplementation is highly effective in increasing its levels within the bloodstream and within cells of the immune system in humans and other animals (Wu *et al.*, 2009).

21.3 Utilization of arginine by multiple pathways

21.3.1 Utilization of arginine by cells in the immune system

Macrophages, lymphocytes and other cell types in the immune system contain Mn^{2+} -dependent arginase (type I and type II) and inducible NO synthase (iNOS; also known as NOS1) to initiate Arg degradation (Wu and Morris, 1998). Expression of constitutive NOS in cells of the immune system is nearly absent, but expression of iNOS in both macrophages and leukocytes is greatly stimulated by endotoxins and pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α , interleukin (IL)-4 and interferon (IFN)- γ (Wu *et al.*, 1999; Morris, 2007). Expression of type I- and type II-arginases is also enhanced by cyclic adenosine monophosphate (cAMP) and the hormones (e.g., glucocorticoids) that increase cAMP production from adenosine triphosphate (ATP) by adenylate cyclase (Morris, 2007), but glucocorticoids suppress expression of both iNOS and guanosine triphosphate (GTP) cyclohydrolase I [a key enzyme in *de novo* synthesis of (6R)-5,6,7,8-tetrahydrobiopterin (BH4), an essential co-factor of NOS] in macrophages and other cell types of the immune system (Shi *et al.*, 2004).

In addition to Arg, iNOS requires O_2 , BH4, NADPH, calmodulin, FMN and FAD to generate NO. L-citrulline, a co-product of iNOS, is readily recycled into Arg at the expense of aspartate and ATP in both resting and activated cells of the immune system via argininosuccinate synthase and argininosuccinate lyase (Wu and Brosnan, 1992). Activities of arginase and iNOS, as well as argininosuccinate synthase and argininosuccinate lyase, depend on physiological or pathological states, but can be markedly enhanced in nearly all cell types under the conditions of various immunological challenges (Wu and Morris, 1998). This explains why concentrations of Arg in plasma are reduced in response to sepsis, infection, and inflammation (Li *et al.*, 2007).

The major products of Arg catabolism in macrophages and leukocytes via the arginase pathway include urea, ornithine, polyamines and proline, whereas iNOS produces relatively large amounts of NO that are cytotoxic to target cells (e.g., tumors and pathogens) (Wu *et al.*, 2009). Polyamines are necessary for initiating and sustaining the proliferation of activated T-lymphocytes and B-lymphocytes.

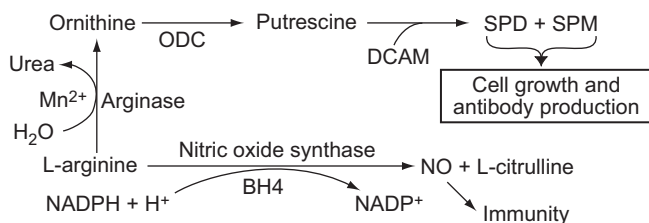


Fig. 21.3 Competition of arginase and NO synthase for arginine in mammalian cells (including immunocytes). Nearly all types of mammalian cells can simultaneously use arginine via the arginase and NO synthase pathways. Amounts of ornithine and NO formed from arginine depend, in part, on the relative activities of these two enzymes, but can be greatly affected by the availability of co-factors. BH₄, tetrahydrobiopterin; DCAM, decarboxylated 5-adenosylmethionine; NO, nitric oxide; ODC, ornithine decarboxylase; SPD, spermidine; SPM, spermine.

Both iNOS-derived NO and proline are critical for macrophage- and T cell-mediated wound healing (Wu *et al.*, 2009).

Arginase and iNOS compete for Arg in all cell types (including microorganisms) as a mechanism to mediate immune responses (Wu and Morris, 1998; Das *et al.*, 2010). Therefore, relative changes in the activities of these two enzymes are the major determinants of NO and polyamine production in the cells (Blachier *et al.*, 2011). Consequently, a marked elevation in arginase activity can result in enhanced synthesis of polyamines but reduced generation of NO (Wu and Morris, 1998). This provides a mechanism responsible for: (i) the survival and growth of immunologically-challenged parasites (Gaur *et al.*, 2007), bacteria (Gobert *et al.*, 2001) and viruses (Zaffuto *et al.*, 2007) in the hosts; and (ii) the proliferation of T-lymphocytes and B-lymphocytes, as well as the production of specific antibodies by B-lymphocytes (Fig. 21.3). Recently, Qualls *et al.* (2010) have shown that mycobacteria use a component of the Toll-like receptor (Myd88) to induce expression of arginase type I in infected macrophages through an autocrine–paracrine mechanism that involves macrophage-produced cytokines. As a result, the macrophages fail to produce sufficient amounts of NO by iNOS to kill the mycobacteria (Qualls *et al.*, 2010). The advanced knowledge about the use of arginase by some pathogens as a means of immune evasion provides a new metabolic basis for preventing and treating many infectious diseases. Besides iNOS, the regulation of expression of argininosuccinate synthase and argininosuccinate lyase is also a strategy to maintain intracellular concentrations of Arg for sustaining NO production in activated macrophages (Wu and Brosnan, 1992).

The K_m values of NOS for Arg are 3–20 μM ($\leq 10\%$ of intracellular Arg concentrations), depending on isoforms (Wu and Morris, 1998). However, increasing extracellular concentrations of Arg from 0.05 to 10 mM dose-dependently increases NO production by endothelial cells and activated macrophages (Wu and Meininger, 2002). It is now clear that Arg promotes the translation of iNOS mRNA in cytokine-stimulated astrocytes (Lee *et al.*, 2003) and BH₄ synthesis in endothelial

cells (Shi *et al.*, 2004), therefore enhancing NO production by inducible and constitutive NOS, respectively. Endogenous metabolites of protein-bound Arg, N^G-monomethyl-L-arginine and asymmetric dimethylarginine, are competitive inhibitors of all the isoforms of the NOS ($K_i = 1.0\text{--}1.6\ \mu\text{M}$) (Alderton *et al.*, 2001). Thus, proper balance of Arg catabolism via the arginase and NOS pathways is necessary to maintain the health of humans and other animals in response to immunological challenges.

21.3.2 Utilization of arginine by other cell types

Because Arg catabolism by non-immune cells affects the availability of Arg for immune cells in the body, it is important to understand how Arg is utilized by non-immune cells. These Arg-degrading pathways are initiated by two isoforms of arginase, three isoforms of NOS, Arg:glycine amidinotransferase and Arg decarboxylase to generate ornithine, NO, creatine and agmatine, respectively (Wu *et al.*, 2009). In mammals, the arginase pathway is quantitatively most important for Arg catabolism. As noted above, type-I arginase (a cytosolic enzyme) is expressed abundantly in hepatocytes and, to a limited extent, in extrahepatic cells, including enterocytes of postweaning mammals, endothelial cells, mammary epithelial cells and red blood cells (only in primates) (Wu and Meininger, 1993; Morris, 2007). In contrast, type-II arginase is widely expressed at relatively low levels in virtually all mitochondria-containing extrahepatic cells and plays an important role in regulating the synthesis of NO, proline and polyamines (Wu *et al.*, 2009). Like the three isoforms of the NOS, arginases I and II are encoded by different genes and differ in their biochemical and immunological properties (Morris, 2007). This results in the production of ornithine and NO from Arg in a cell- and tissue-specific manner. A salient observation from recent studies is that arginase is released from injured cells and tissues into extracellular fluid to hydrolyze Arg to ornithine plus urea (Reid *et al.*, 2007; Wu *et al.*, 2008), causing reductions in concentrations of Arg in plasma and wounds. This problem can be ameliorated by specific inhibitors of arginase (e.g., N- ω -hydroxy-nor-L-arginine; 2(S)-amino-6-boronohexanoic acid; S-(2-boronoethyl)-L-cysteine; and N^G-hydroxy-L-arginine) (Morris, 2009). High concentrations of some amino acids (e.g., lysine and branched-chain amino acids) also inhibit arginase activity (Wu and Morris, 1998; Meininger and Wu, 2002). Furthermore, lysine inhibits Arg uptake by all cell types (Wu, 2013), thereby attenuating NO production by iNOS and other NOS isoforms.

Large amounts of Arg (e.g., 2.3 g/day in a 70 kg man) are utilized for the production of creatine in the body via the inter-organ cooperation of kidneys, pancreas, liver and skeletal muscle (Wu and Morris, 1998). In the form of phosphocreatine, creatine is a major storage of ATP in the brain and skeletal muscle. Additionally, creatine has an antioxidant function (Fang *et al.*, 2002), reduces inflammatory responses (Bassit *et al.*, 2008) and improves glucose tolerance (Gualano *et al.*, 2008) in humans. Thus, endogenous synthesis of creatine is critical for neurological and muscle development and function (Beal, 2011;

Tarnopolsky, 2011). This, in turn, can profoundly impact the immune responses in the host (Wu, 2009). Because the methylation of guanidinoacetate to form creatine consumes more methyl groups than all other methylation reactions combined, creatine synthesis from Arg regulates the availability of the methyl group donor for other methylation reactions, such as the formation of methionine from homocysteine (Brosnan *et al.*, 2011). Thus, Arg can play a role in regulating one-carbon unit metabolism in the whole body (Wang *et al.*, 2012).

21.4 Roles of arginine in immune responses

21.4.1 Role of arginine in the innate immune system

An ability of the host to prevent the invasion of various pathogens depends on both the innate (natural, non-specific) and the acquired (adaptive, specific) immune systems. The innate immune system consists of several integral components: (i) physical barriers (e.g., skin, endothelial cell layer in respiratory tract and gastrointestinal tract); (ii) mononuclear phagocytes (e.g., monocytes and macrophages), dendritic cells, polymorphonuclear granulocytes (e.g., neutrophils, eosinophils and basophils), mast cells, natural killer (NK) cells and platelets; (iii) humoral factors, including collectins, complements, lysozymes, C-reactive proteins and interferons (Field *et al.*, 2000; Li *et al.*, 2007); (iv) antimicrobial peptides in the mucosa and lumen of the small intestine (Sherman *et al.*, 2006); and (v) neutrophil extracellular traps, comprising DNA and proteins as major structural components (Buchanan *et al.*, 2006). The innate immune system can rapidly respond to invading microbes and is the first line of defense against infections. However, the major limitations of the innate immune system include non-specificity and lack of a memory effect (Calder, 1995).

Available evidence shows that physiological levels of Arg are essential to the functions of the innate immune system via mechanisms that involve: (i) synthesis of NO and reactive oxygen species; (ii) antimicrobial activity; (iii) secretion of hormones (e.g., insulin, growth hormone, prolactin and insulin-like growth factor-I) that regulate the metabolism and activity of immunocytes; and (iv) signal transduction pathways via protein kinases (Li *et al.*, 2007). For example, NO synthesis by iNOS in macrophages and neutrophils is necessary and sufficient to kill viruses, bacteria, fungi, malignant cells, intracellular protozoa and parasites in mammals, birds, terrestrial animals, low vertebrates (e.g., freshwater and marine fishes) and invertebrates (e.g., shrimp) (Hibbs *et al.*, 1987; Bronte and Zanovello, 2005). Additionally, dietary supplementation with Arg beneficially affects the concentrations of leukocytes in the blood of weanling young mammals (Tan *et al.*, 2009). Notably, Mieulet *et al.* (2010) have shown that reducing the concentration of extracellular Arg can compromise the innate immune response by impairing a specific MAPK pathway down-stream of the Toll-like receptor 4 signaling. In this signal transduction pathway, Arg prevents the dephosphorylation of tumor-promoting locus 2 (TPL-2; a MAPK kinase) via yet unknown mechanisms, leading to the activation of MAPK and the subsequent production of pro-inflammatory

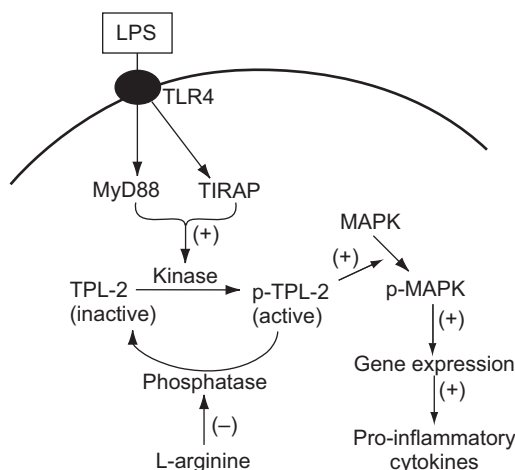


Fig. 21.4 An important role for L-arginine to promote innate immune responses by preventing the phosphorylation of a specific mitogen-activated protein kinase pathway downstream of the Toll-like receptor 4 signaling in immune cells. LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases (e.g., extracellular signal-regulated kinases 1 and 2 (ERK 1/2)); TLR4, Toll-like receptor 4 (denoted by a solid circle); TPL-2, a specific MAPK kinase.

cytokines (Fig.21.4). Thus, Arg has been considered a master and commander of innate immune responses (Morris, 2010).

21.4.2 Role of arginine in the acquired immune system

The adaptive (acquired) immune system consists of T-lymphocytes, B-lymphocytes and humoral factors. At birth, this immune system is largely present but functionally immature (Michaëlsson *et al.*, 2006). The bone marrow is primarily responsible for hematopoiesis and lymphopoiesis, while the thymus is required for T-cell development. Secondary lymphoid organs include the spleen, lymphoid nodes and the mucosa-associated lymphoid tissues in the gastrointestinal, respiratory and reproductive tracts. Because each lymphocyte carries surface receptors for a single antigen, the acquired immune response is highly specific. In contrast to the innate immune system, the adaptive immune system becomes effective over days after initial stimulation and possesses immunological memory through the production of specific antibodies by B-lymphocytes. When pathogens escape the humoral immunity, they are targeted by cell-mediated immunity that involves the production of cytokines (e.g., IFN- γ) and other cytotoxic proteins by T-lymphocytes (Calder and Yaqoob, 1999).

Physiological levels of Arg regulate the adaptive immune system via mechanisms that involve: (i) maturation and proliferation of T-lymphocytes and B-lymphocytes; (ii) production of cytokines and specific antibodies by T-lymphocytes and B-lymphocytes, respectively; (iii) circulating levels of anabolic hormones; and (iv) expression of T-cell receptors (Li *et al.*, 2007). For example, as

a substrate for enzymes that catalyze the synthesis of proteins and polyamines, Arg is required for the maximal proliferation of rodent and human T-lymphocytes in response to mitogens (Field *et al.*, 2002). In mice overexpressing type-I arginase in the small intestine, the resulting deficiency of Arg impaired the development of progenitor-B to precursor-B lymphocytes in the bone marrow and decreased the number of B-lymphocytes in secondary lymphoid organs (De Jonge *et al.*, 2002). Importantly, these immunological defects were effectively reversed by subcutaneous administration of Arg (De Jonge *et al.*, 2002). Additionally, growth hormone increases the production of T-lymphocytes in the thymus, the number of hematopoietic progenitor cells in the bone marrow, the response of T cells to cytokines and the antigen-presenting capability of dendritic cells in an Arg-dependent manner (Calder and Yaqoob, 2004; Li *et al.*, 2007). Also, Arg stimulates the synthesis and expression of the T-cell receptor ζ chain (CD3 ζ) that is required for T-cell receptor integrity (Rodriguez *et al.*, 2003; Bansal *et al.*, 2004). Furthermore, dietary supplementation with 1% or 2% Arg to healthy rodents or tumor-bearing or septic rats increased thymic weight, the number of thymic lymphocytes, T-lymphocyte proliferation, the cytotoxicity of specific cells (T-lymphocyte, macrophages and NK cells), (IL)-2 production, IL-2 receptor expression on T lymphocytes and the delayed type hypersensitivity response (Calder and Yaqoob, 2004). Similar results have been reported for weanling piglets (Tan *et al.*, 2009).

Thus, Arg profoundly affects the adaptive immune system in humans and other animals. In support of this notion, clinical studies have shown that enteral or parenteral provision of Arg (e.g., 8–20 g per day; corresponding to 1.5–3.6 times the Arg intake by an average adult) improves immune functions and clinical outcomes in patients with burn injury, cancer, HIV infection, major traumas and gastrointestinal surgical operations (Field *et al.*, 2002; Suchner *et al.*, 2002). The benefits are indicated by enhanced T-cell function, increased antibody production, accelerated wound healing mediated by immune cells or a reduction in infection, ventilator days and intensive care unit and hospital stay.

21.4.3 Role of arginine in autoimmunity

Autoimmune disease results from the destruction of host cells and tissues by activated host immunocytes (e.g., macrophages and T-lymphocytes). The pathogenesis is mediated in part by high concentrations of free radicals (e.g., NO and superoxide) and other reactive oxygen species (Peranzoni *et al.*, 2007). NO is an oxidant and inhibitor of enzymes that contain an iron–sulfur center, and that high levels of NO rapidly react with H_2O_2 to form peroxynitrite (ONOO $^-$) (Fang *et al.*, 2002). NO and peroxynitrite oxidize biomolecules (e.g., proteins, amino acids, lipids and DNA), which leads to cell injury and death. Thus, large amounts of NO produced by iNOS can exert a deleterious effect on mammalian cells and contribute to the pathogenesis of many autoimmune diseases, including type-I diabetes mellitus, rheumatoid arthritis, multiple sclerosis, interstitial nephritis, inflammatory bowel disease and encephalomyelitis (Flynn *et al.*, 2002). Under these

conditions, Arg supplementation could ‘fuel the fire’ to worsen clinical outcomes and, therefore, should be avoided. Inhibition of NO synthesis from Arg by iNOS may help prevent the development of autoimmune disease, including type-I diabetes mellitus (Wu, 1995).

21.5 Mechanisms by which arginine modulates immune function

21.5.1 Nitric oxide, carbon monoxide and hydrogen sulfide

Growing evidence shows that Arg modulates immune responses by regulating the generation of three signaling gases: NO, carbon monoxide (CO), and hydrogen sulfide (H₂S) (Bhatia, 2010; Blancou *et al.*, 2011). CO and H₂S are synthesized from heme and cysteine by heme oxygenase (HO) and cystathionine β -synthase (CBS)/cystathionase (CSE), respectively, in many cell types including immunocytes. These synthetic pathways are regulated by Arg (Li *et al.*, 2009c). For example, dietary supplementation with Arg stimulates HO3 expression in white adipose tissues of Zucker diabetic fatty rats (a genetically obese type-II diabetic animal model) (Fu *et al.*, 2005) and in diet-induced obese rats (Jobgen *et al.*, 2009). Notably, a common finding in these two animal models in response to Arg supplementation is enhanced expression of the HO3 gene in the fat pad, despite their marked differences in global expression of adipose tissue genes. Additionally, Arg upregulates HO1 expression and CO generation in cells likely through an NO-dependent mechanism (Chang *et al.*, 2008). In addition to CO synthesis, administration of Arg to rats with high blood flow-induced pulmonary hypertension increased: (i) CSE expression in smooth muscle cells of the pulmonary arteries; (ii) concentrations of H₂S in plasma; and (iii) H₂S production by the lungs (Yanfei *et al.*, 2006). Moreover, NO upregulates CSE expression and H₂S synthesis in vascular smooth muscle (Zhao *et al.*, 2001).

High concentrations of NO, CO and H₂S inhibit microbial growth and kill pathogenic organisms (Nobre *et al.*, 2007; Zhi *et al.*, 2007; Zhang and Bhatia, 2008). In contrast, physiological levels of these gases have potent cytoprotective effects on immunocytes. NO and CO bind the heme group of soluble guanylate cyclase, thereby activating this enzyme for the generation of cyclic guanosine monophosphate (cGMP) from GTP (Fig. 21.5). cGMP triggers cGMP-dependent kinases and the phosphorylation of target proteins that elicit a series of physiological responses, including the cGMP-mediated pathways that regulate inflammatory responses in immune and central nervous systems (Shenoy and Agarwal, 2010; Pifarre *et al.*, 2011). Physiological levels of CO decrease the circulating levels of pro-inflammatory cytokines (e.g., IL-1 β and -6, and TNF- α) (Brusko *et al.*, 2005; Ning *et al.*, 2005; Kim *et al.*, 2008), while increasing the production of anti-inflammatory cytokines (e.g., IL-10) via the p38 MAPK pathway (Dolinay *et al.*, 2004). H₂S can stimulate the generation of cAMP from ATP by the plasma membrane-bound adenylate cyclase (Li *et al.*, 2009c). The cAMP-dependent

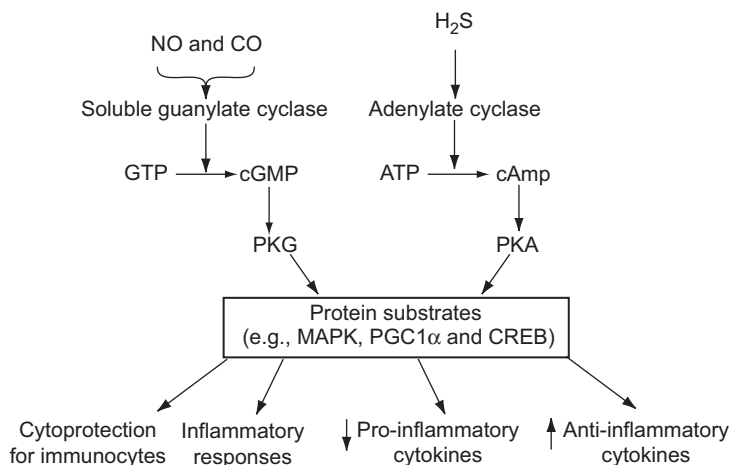


Fig. 21.5 Gaseous signaling in immunocytes to affect immune responses. Nitric oxide (NO)/carbon monoxide (CO) and hydrogen sulfide (H_2S) activate soluble guanylate cyclase and plasma membrane-bound adenylylate cyclase, respectively, to generate cGMP and cAMP. Through the phosphorylation of protein kinase G (PKG) and protein kinase A (PKA), elevated concentrations of cGMP and cAMP activate specific kinases (including mitogen-activated protein kinases (MAPK)), resulting in a series of physiological responses to affect immune function. CREB, cAMP response element-binding protein; PGC1 α , peroxisome proliferator-activated receptor γ co-activator-1 α .

protein kinase A phosphorylates proteins to affect inflammatory bowel disease (Fig. 21.5), including ulcerative colitis and Crohn's disease (Hosseini-Tabatabaei and Abdollahi, 2008). Thus, an inhibition of phosphodiesterase-catalyzed hydrolysis of cGMP and cAMP may be beneficial for the management of autoimmune disease (Shenoy and Agarwal, 2010).

21.5.2 mTOR signaling

Proliferation of lymphocytes and production of specific antibodies by B-lymphocytes critically depend on intracellular protein synthesis and degradation (Miyamoto *et al.*, 2000). It is now known that mTOR is the master positive regulator of protein synthesis in immunocytes (Salmond *et al.*, 2009) and also an inhibitor of lysosomal proteolysis via autophagy (Meijer and Dubbelhuis, 2004). mTOR is a highly conserved serine/threonine protein kinase, also known as FK506 binding protein 12-rapamycin associated protein 1. The mTOR system consists of (i) rapamycin-sensitive complex 1 (mTOR1) [mTOR, raptor (regulatory associated protein of TOR), and G protein b-subunit-like protein] which can be activated by Arg; and (ii) rapamycin-insensitive complex 2 (mTOR2) [mTOR, rictor (rapamycin-insensitive companion of TOR), MAPK-associated protein 1, and G protein b-subunit-like protein]. These two complexes are structurally and functionally distinct in cells (Suryawan and Davis, 2011). mTOR1 phosphorylates 4E-BP1 (eIF4E-binding protein-1) and ribosomal protein S6 kinase-1 (S6K1),

resulting in initiation of protein synthesis and possibly inhibition of autophagy (Meijer and Dubbelhuis 2004; Yao *et al.*, 2008). mTOR2 phosphorylates protein kinase B/Akt and may function to regulate cell proliferation, differentiation and migration and cytoskeletal reorganization (Bazer *et al.*, 2012). Results of *in vitro* studies have revealed that Rag GTPases bind raptor and, therefore, mediate amino acid signaling to mTORC1 (Sancak *et al.*, 2008). There is evidence that mTOR regulates: (i) the activation, as well as proliferation, differentiation and cell-fate determination of CD8 T cells (Miyamoto *et al.*, 2000; Salmond and Zamoyska, 2010); (ii) T-lymphocyte trafficking (Sinclair *et al.*, 2008); and (iii) the production of antibodies by B-lymphocytes (Zhang *et al.*, 2011). It is imperative that *in vivo* studies be conducted with animal models to verify the *in vitro* discoveries (Wu, 2010a,b).

21.6 Chemical forms and safety of arginine supplementation

21.6.1 Chemical forms of arginine

When dietary protein is hydrolyzed in the lumen of the gastrointestinal tract and when intracellular protein is degraded via multiple pathways, the free base form of Arg is generated (Wu, 2009). This form of Arg accepts a proton, resulting in an elevation of pH within a physiological range. Due to the presence of HCl in the stomach fluid, Arg released from appropriate amounts of dietary protein (e.g., up to 1 and 2 g protein/kg body weight per day for adult humans and children, respectively) does not disturb acid–base balance in the body (Wu *et al.*, 2009). Likewise, results of studies from growing and pregnant swine indicate that supplementing 0.4 to 0.8% Arg (free base or Arg-HCl form) to the basal diet containing 0.7 to 1% Arg does not affect pH in the lumen of the gastrointestinal tract or plasma (Wu *et al.*, 2013). However, Arg-HCl in a neutral solution (e.g., saline) should be used for intravenous administration into humans or other animals to prevent acid–base imbalance (Lassala *et al.*, 2010, 2011). At present, commercially available Arg products for oral administration include Arg (free base form), Arg-HCl and Arg- α -ketoglutarate.

21.6.2 Safety of arginine supplementation

Arg is stable under sterilization conditions (e.g., high temperature and high pressure) and is not toxic to cells (Flynn *et al.*, 2002). Thus, its administration at an appropriate dose, chemical form and means is safe for humans and other animals. For example, neonatal pigs, growing-finishing pigs, pregnant pigs and adult rats tolerated large amounts of chronic supplemental Arg-HCl (at least 0.62, 0.32, 0.21 and 2.14 g Arg/kg body weight per day, respectively) administered via enteral diets without any adverse effects (Wu *et al.*, 2007a). Additionally, long-term intravenous infusion of Arg-HCl to ewes at 81 mg/kg body weight per day between days 60 and 147 (term) of gestation was safe for both mother and fetus (Wu *et al.*, 2007a). Furthermore, intravenous Arg infusion (up to 0.5 g Arg-HCl/

kg body weight for infants, or 30 g Arg-HCl for adults over 30–60 minutes) or oral Arg (9 g Arg-HCl/day for adults) has no adverse effects on humans (Wu and Meininger, 2000; McNeal *et al.*, 2010). However, higher oral doses of Arg-HCl (9 g/day) are occasionally associated with nausea, gastrointestinal discomfort, and diarrhea for some subjects (Grimble, 2007), which may result from a rapid and excess production of NO by the gastrointestinal tract and from impaired intestinal absorption of other dietary basic amino acids (lysine and histidine). A solution to this potential problem may be the alternative use of L-citrulline, a precursor for arginine synthesis (Wu and Meininger, 2000). Finally, because lysine competes with Arg for entry into cells (Wu and Morris, 1998), the ratios of Arg to lysine in diets can profoundly influence the physiological effect of Arg supplementation and should be less than 3.0:1 (g/g).

21.6.3 Additional cautions regarding arginine supplementation

While Arg can be used to prevent or ameliorate endothelial dysfunction, enhance foetal and neonatal growth, and increase resistance to infectious disease in humans and animals, additional precautions should be taken when considering dietary supplementation with Arg. For example, because excessive production of NO may be harmful to cells, it would not be advisable to administer Arg alone to animals or patients with severe infections, active inflammatory or autoimmune disorders, active malignancy (e.g., late stages of tumorigenesis) or pathological angiogenesis. Also, as with other nutrients (e.g., glucose, n-3 polyunsaturated fatty acids, vitamins and minerals), improper use of Arg (e.g., high dose, disturbance of acid–base balance and antagonism among basic amino acids) may yield an undesirable effect and should be avoided in dietary supplementation and clinical therapy. Therefore, it is important that Arg be taken in divided doses on each day of administration (i) to prevent gastrointestinal tract disorders due to abrupt production of large amounts of NO; (ii) to increase the availability of circulating Arg within physiological ranges over a longer period of time; and (iii) to avoid a potential imbalance among amino acids (Wu *et al.*, 2007a).

21.7 Conclusion and future trends

Arginine is an essential nutrient for both innate and adaptive immune systems. It serves as a physiological substrate for enzymes, a building block for tissue proteins and a regulatory molecule in signal transduction pathways in cells (including immunocytes). There is complex compartmentalization of Arg degradation at cellular, tissue and whole-body levels, which necessitates thoughtful considerations of dietary requirement of Arg by adults and neonates, the chemical form and dose of supplemental Arg, the composition of amino acids in the basal diet, and the health status of the subjects (Wu *et al.*, 2009, 2012). Based on multiple pathways for Arg utilization at high rates, dietary Arg supplementation may be a necessary strategy to maintain Arg homeostasis for good health and body functions under

many physiological and pathological conditions (Tan *et al.*, 2012; Dai *et al.*, 2013). An important outcome of this research is the current availability of Arg-supplemented 'immunonutrition' for humans (Kudsk, 2006) and feed-grade Arg for livestock species. Animal studies have consistently shown that chronic enteral or parenteral administration of appropriate doses of Arg is safe for neonates, young adults and gestating mothers (Wu *et al.*, 2009). However, physiological tolerance to long-term Arg supplementation in humans remains a major concern due to a lack of clinical data. Much research is needed to address this critical issue so as to fully realize the health benefits of dietary supplementation with Arg as a functional amino acid in both humans and other animals.

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22

Sulphur amino acids and immune functions

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Abstract: This chapter describes the metabolic background to the dietary requirements for the sulphur amino acids methionine and cysteine and the interrelationships between their metabolism and that of early harbingers of immune activation; methyl-metabolism and polyamine synthesis. The chapter describes clinical situations in which sulphur amino acids may become conditionally essential (e.g. HIV/AIDS, cancer, arthritis) and may require supplementation with these amino acids or their analogues.

Key words: S-adenosylhomocysteine, S-adenosylmethionine, liver disease, critical illness, HIV/AIDS, reactive oxygen species, glutathione.

22.1 Introduction

The dominant concept in protein nutrition is that some amino acids are essential (indispensable) and others are not. More specifically, essential amino acids 'cannot be synthesized by the animal organism, out of materials ordinarily available to the cells, at a speed commensurate with the demands for normal growth' (Borman *et al.*, 1946) whereas the non-essential amino acids can be synthesized at an adequate rate.

This simple but subtle classification was devised by William Rose, and others, in the 1930s from a series of experiments on rats and, later, humans, consuming synthetic diets lacking in individual amino acids. In rats, the test of adequacy was that normal growth and development was maintained. In humans, an amino acid was considered essential if its exclusion led to loss of appetite and weight and failure to maintain nitrogen balance at adequate nitrogen intake. Additionally,

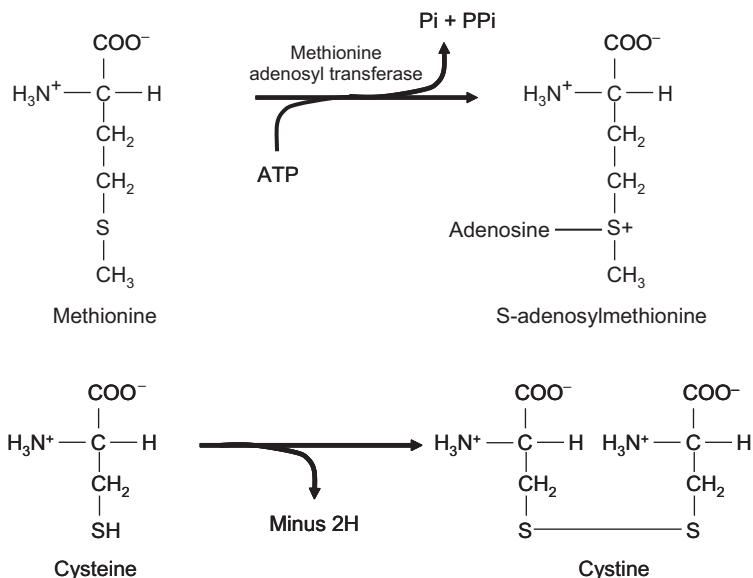


Fig. 22.1 Structure of principal sulphur amino acids. Methionine adenosyl transferase catalyses the addition of the adenosine moiety to form a highly reactive sulphonium ion which will donate the methyl group to a suitable nucleophilic centre (e.g. $-N:$). Cysteine can be oxidized to form the disulphide form, cystine.

subjects often showed 'nervous irritability' and felt extremely fatigued (Rose, 1968). In one set of experiments, subjects consumed the synthetic diets as lemon-flavoured drinks and baked wafers which differed in DL-methionine content with or without varying amounts of cysteine, for example. How Rose and his colleagues maintained the motivation and compliance of the loyal volunteers is a mystery since each subject might sequentially consume nine different diets for a period of five to seven days (Rose and Wixom, 1955). Another biological impairment associated with exclusion of an essential amino acid was reduced spermatogenesis, a marker of profoundly reduced cellular proliferation. In the case of the two sulphur amino acids (Fig. 22.1), removal of the large part of methionine led to signs of deficiency which could be partly relieved by dietary cysteine; whilst deficiency of cysteine could be partly relieved by methionine (Rose and Wixom, 1955). Animals fed a synthetic diet lacking cysteine and methionine stopped growing, but this resumed if homocysteine and a source of methyl groups (e.g. choline) were added back to the diet (du Vigneaud *et al.*, 1939, 1950). In other words, both Rose and du Vigneaud recognized that methionine was an essential amino acid which could be produced from its component parts if they were given together. Furthermore, the requirement for methionine from which cysteine is synthesized (Fig. 22.2), could be reduced if excess cysteine were given, but only up to a certain point. Under the abnormal circumstances of du Vigneaud's experiments, methionine could not be synthesized 'at a rate fast enough for the demands of the young growing rat' (du Vigneaud *et al.*, 1950).

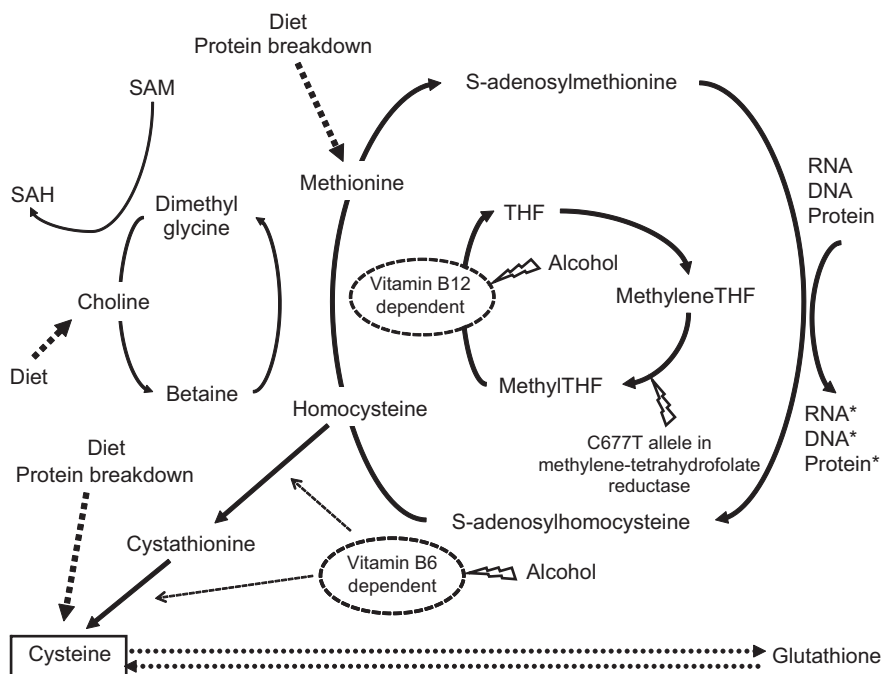


Fig. 22.2 Metabolism of sulphur amino acids – transmethylation and trans-sulphuration. Further metabolism of methionine occurs through conversion to S-adenosylmethionine (SAM) which is the principal donor of methyl groups to RNA, DNA and protein. S-adenosylhomocysteine (SAH) loses its adenosyl group to form homocysteine (Hcy). Hcy is the precursor for cysteine synthesis. Remethylation of Hcy occurs from dietary methyl groups (choline) or from endogenous one-carbon groups. Alcohol abuse specifically targets remethylation of Hcy and its conversion to cysteine. THF, tetrahydrofolate.

Subsequently, Finkelstein and colleagues confirmed that if methionine were removed from the diet and substituted with cystine, less cysteine was synthesized by the liver; cellular concentrations of methionine's intermediates were reduced, but methionine metabolism was maintained as long as there was sufficient dietary choline (Finkelstein, Martin, and Harris, 1988). In humans, only a lesser proportion of methionine can be replaced by cystine (Raguso *et al.*, 1997; Fukagawa *et al.*, 1998) and this is a contentious issue for two reasons. In metabolic terms, it is not clear how much methionine can be replaced by cystine at each stage of the human lifecycle, especially in relation to artificial nutrition support (Ball *et al.*, 2006). The nutritional issue concerns the adequacy of diets which contain poor quality protein. This is described by their Protein Digestibility-Corrected Amino Acid Score (PDCAAS) which is calculated from protein digestibility and individual scores for essential amino acids (e.g. methionine), and not on scores for those amino acids which might become conditionally essential during illness (e.g. cystine).

Weaning foods may contain protein from milk and vegetables which contain

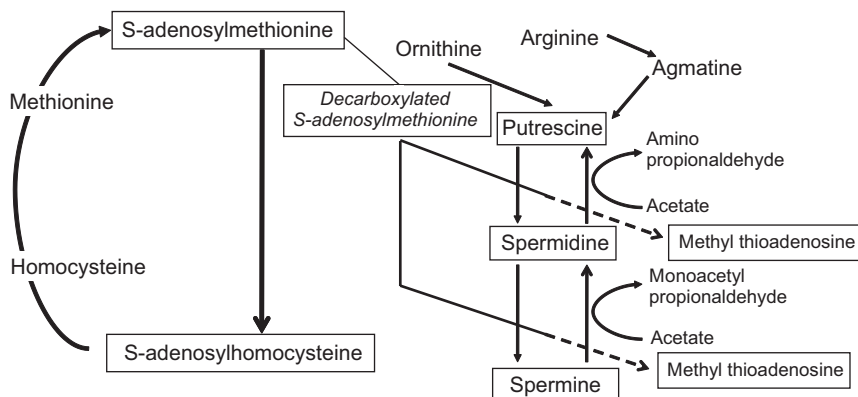


Fig. 22.3 S-adenosylmethionine as portal for polyamine synthesis. The carbon skeleton of methionine in SAM is released from decarboxylated SAM by SAM decarboxylase (SDC) in the form of propylamino groups and donated to the growing carbon chain of the polyamines. Ornithine is decarboxylated to putrescine by ornithine decarboxylase and initiates this process. New synthesis of SDC and ODC occurs when growth is stimulated and both enzymes are promising targets for inhibitors in cancer chemotherapy.

adequate and inadequate amounts of sulphur amino acids, respectively (Schaafsma, 2005). If the situation is imagined of a poorly nourished child in a developing country who is subject to repeated bouts of respiratory or gastrointestinal infection, then the stunting and progressive relative loss of muscle protein this causes may help to meet requirements for the sulphur amino acids for synthesis of acute-phase proteins (Reeds *et al.*, 1994) but not for the extra liver glutathione production which is required in kwashiorkor, for example (Jahoor *et al.*, 2006a,b). This is one reason why supplements of glutathione itself, or of N-acetylcysteine (NAC), have been given to severely malnourished children admitted to hospital for rehabilitation in order to boost intracellular hepatic antioxidant defences (Becker *et al.*, 2005).

The concept behind this is that some amino acids which are non-essential may become so when their supply or synthesis is rate-limiting, under certain clinical conditions (Grimble, 1993; Reeds, 2000). During artificial nutrition support, for example, it has not always been possible to add sufficient cysteine, tyrosine or glutamine to intravenous solutions because of their low solubility or poor stability. Alternatively, under sustained oxidant attack during critical illness, synthesis rates of antioxidant glutathione might be poor because there is low dietary availability of its constituent amino acids cysteine, glutamine and glycine (Jackson *et al.*, 1987).

The metabolism of methionine and cysteine is complex (Figs 22.2 and 22.3) and temporary distortions of the metabolic pattern may appear during elaboration of the different parts of the injury response. Conversely, supplementation with sulphur amino acids or their metabolites or even inhibition of their metabolism has been shown to be clinically useful and this will be discussed. The reader is directed elsewhere for an overview of amino acid requirements in general (Harper and Yoshimura, 1993; Millward, 1998; Reeds, 2000).

22.2 Methionine metabolism

Methionine shows considerable metabolic versatility through its engagement in two metabolic cycles and several synthetic pathways (Fig. 22.2). Firstly, it is incorporated into newly synthesized protein, being returned to the body intracellular pool after release by protein breakdown. For methionine, this pathway dwarfs remethylation or trans-sulphuration by a factor of 3:1 and 10:1, respectively (MacCoss *et al.*, 2001). Methionine also carries a methyl group which is the substrate for most biological methylations. The 'activated' form of methionine, S-adenosylmethionine (SAM) owes its potency to the electrophilic sulphonium ion which allows nucleophilic groups (i.e. N: and O:) in protein amino acids or the nucleobases of RNA to abstract the methyl group from SAM (Loenen, 2006). After donation of the methyl group, S-adenosylhomocysteine (SAH) is hydrolysed to release adenosine and homocysteine (Hcy) which has two possible fates; it can either be remethylated to methionine or can enter the trans-sulphuration pathway to form cystathionine and, ultimately, cysteine (Brosnan *et al.*, 2007; Grillo and Colombatto, 2008).

The two key features of the SAM–SAH cycle are therefore firstly, that it is the only route by which methionine can be transformed into other compounds and secondly, the cycle of methylation–remethylation only consumes methyl groups, not methionine, and is theoretically self-perpetuating as long as methyl donors are supplied (Fig. 22.2). Methionine degradation occurs after it has been adenosylated to SAM through two routes (Hoffer, 2002). Firstly, S-adenosylmethionine decarboxylase (SDC) will produce decarboxylated SAM (dcSAM – see Fig. 22.3), the precursor for the aminopropyltransferases which transfer the aminopropyl group successively to the chain-lengthening cascade of polyamines (Pegg, 2009). Secondly, successive conversion of methionine to SAM, SAH and Hcy will make it available for cysteine synthesis before ultimate oxidation to sulphate (Stipanuk and Ueki, 2011).

It can therefore be seen that any limitation in the rate of remethylation (through a lack of methyl donors or reaction co-factors) will lead to an accumulation of homocysteine whilst at the same time polyamine synthesis necessary for cell division will be hampered (Figs 22.2 and 22.3).

22.3 Cysteine synthesis and metabolism

Homocysteine is trans-sulphurated with serine in visceral tissue to produce cystathionine and then cysteine, which can engage in two metabolic cycles and two other metabolic pathways (Fig. 22.2).

- Cysteine is a protein amino acid and will be incorporated into newly synthesized protein and be released by protein breakdown as part of a cycle.
- Cysteine is a constituent amino acid of the antioxidant tripeptide, glutathione (GSH) and will be released when glutathione is degraded (Biolo *et al.*, 2007).
- Cysteine can be irreversibly converted to cysteine sulphinic acid through the action of cysteine dioxygenase (Stipanuk *et al.*, 2009). This key step in disposal of

excess cysteine is tightly regulated by methionine and cysteine availability. Cysteinesulphinate may either be converted to pyruvate and sulphate (excreted in urine) or to taurine which has other useful functions (e.g. as an antioxidant (Franconi *et al.*, 2006)).

The magnitude of these processes has been estimated by isotopic labelling methods in healthy humans (Fukagawa *et al.*, 1998; MacCoss *et al.*, 2001). The majority of methionine flux is engaged in protein metabolism (61.7%) whereas cysteine synthesis accounts for 10.4% and the SAM–SAH cycle comprises 8.5% or 18.7% depending on whether the estimate is for Hcy methylation or SAM transmethylation reactions (MacCoss, *et al.*, 2001). Estimates of the ratio of GSH synthesis to cysteine synthesis vary. Reeds gave a high estimate of 5:1 (Reeds, 2000) but lower ratios of 1:1 can be extrapolated from human and rat isotope studies (Fukagawa *et al.*, 1996; Malmezat *et al.*, 2000). Whichever is correct, the size of these processes means that the balance of cysteine synthesis, protein turnover and the γ -glutamyl cycle (Meister, 1995b) will maintain cysteine homeostasis, and there is an additional buffer because the intracellular concentration of GSH is high; as much as 500 times that of cysteine in red blood cells, for example (Meister, 1995a,b). Additionally, GSH synthesis and breakdown to its constituent amino acids are nearly balanced (Fig. 22.2). A corollary to this is that alterations of GSH metabolism by inflammatory process will have a profound effect on cysteine dynamics, and reductions in GSH concentrations signal a very significant drain on this antioxidant compound. Some examples of this phenomenon are:

- The role of GSH in conjugating toxins which will be excreted in the form of adducts of N-acetylcysteine or as other thiols (Meister, 1995b; Muntane, 2009) which are biomarkers for exposure to compounds as diverse as acrylamide (Huang *et al.*, 2011) or the anaesthetic sevoflurane (Kharasch and Jubert, 1999). This only represents a large drain on the GSH pool in the special case of paracetamol overdose (see below).
- The need for GSH to constantly neutralize reactive oxygen species (ROS) which are produced as a result of the escape of about 2% of electrons from the mitochondrial electron transport chain during normal aerobic metabolism (Ciriolo, 2005). Because of the magnitude of aerobic oxidation, this is not a trivial amount and requires continuous regeneration of GSH from GSSG.
- ROS production increases during critical illness (Singer, 2007); indeed the requirement to maintain a reduced intracellular redox potential is a main cause of conversion of GSH to the oxidized disulphide form, GSSG, in the injured liver (Cesaratto *et al.*, 2004).
- Infection of lymphocytes by HIV may lead to generation of increased ROS which stimulates replication of viral DNA through increased activation of nuclear factor κ B (NF κ B). The failure of GSH antioxidant defences is critical for subsequent virion production and will be discussed later (Nakamura *et al.*, 2002).
- Inflammation, *per se*, leads to an increase in generation of ROS in visceral organs under the influence of cytokine action (e.g. tumour necrosis factor

alpha, $\text{TNF}\alpha$). The ROS are themselves signalling molecules which will stimulate cell antioxidant defences in order to restore redox balance. Additionally, export of GSSG will maintain redox balance if GSH uptake matches it (Lopez-Mirabal and Winther, 2008) but, if this is insufficient, then excessive ROS production leads to activation of cell death pathways and apoptosis (Circu and Aw, 2010).

Thus, during infection or acute illness, the increased requirement for GSH can be met either by increased reduction of GSSG or by increased synthesis of GSH from its precursor amino acids. Inhibition of glutathione synthesis by buthionine sulfoxide leads to increased injury severity and even death in clinical situations as diverse as galactosamine-induced liver injury (Wang *et al.*, 2007), cardiac ischaemia-reperfusion injury (Leichtweis and Ji, 2001), stress-induced gastric injury (Hirota *et al.*, 1989) or paracetamol toxicity (Miners *et al.*, 1984). In each case, the acute-phase response of muscle protein breakdown was not sufficiently rapid to allow release of sufficient cysteine as precursor for glutathione synthesis (Reeds *et al.*, 1994). If an adequate acute-phase response cannot be mounted, because of protein depletion, glutathione synthesis may be insufficient to meet the increased oxidative stress of inflammation, as has been shown in protein-depleted piglets (Jahoor *et al.*, 1995) or in patients with HIV infection (Jahoor *et al.*, 1999).

22.4 S-adenosylmethionine metabolism

22.4.1 Polyamines

SAM is one of the most versatile metabolic sources of precursors for other pathways (Loenen, 2006). It provides methylene, amino and ribosyl groups in addition to a major flux of propylamino groups into polyamine synthesis (Fig. 22.3), all of which represent a drain on both the carbon and methyl moieties of the intracellular pool of SAM (Fontecave *et al.*, 2004). The production of polyamines is essential at the earliest stages of cell division and of organ adaptation (Auvinen *et al.*, 1992; Shantz and Levin, 2007). This pleiotropic process is initiated by the conversion of ornithine to putrescine by ornithine decarboxylase (ODC). It was described, in one of the most important early reviews of the topic, as '*fluctuating rapidly in target tissues in response to hormones and drugs that alter the rate of macromolecular synthesis*' (Russell, 1985).

One mechanism is the rapid expression of ODC mRNA as occurs for example within four hours of UVB light irradiation injury, peaking at 24 hours, whilst the protein signal is rapidly extinguished. The half-life of decay in ODC after cycloheximide inhibition of protein synthesis was estimated to be 24 minutes (Verma *et al.*, 1979). Inhibition of ODC activity by the analogue difluoromethylornithine profoundly affects cellular processes such as intestinal hyperplasia which occurs following pancreatico-biliary diversion (Dowling *et al.*, 1985). Expression of ODC is under very tight control except in the case of transformed cancer cells (Pegg and Feith, 2007). The second step in polyamine

synthesis is catalysed by SDC to produce S-adenosyl-1-(methylthio)-3-propylamine which is the donor of aminopropyl groups to putrescine to form spermidine, and to spermidine to form the higher polyamine, spermine (Loenen, 2006; Pegg, 2009). As in the case of ODC, SDC is tightly controlled by growth factors and also by the levels of polyamines and is rightly considered to be a key target for inhibitors which could make extremely potent anti-cancer drugs (Pegg, 2009). The reader is directed towards some excellent reviews on this topic (Agostinelli *et al.*, 2010; Pegg, 2009).

22.4.2 Free radicals

SAM also generates a powerful free radical, 5'-deoxyadenosine which can remove a proton from a fairly unreactive substrate, such as a C–H bonds in an alkyl group and thus initiate a radical reaction (Wang and Frey, 2007; Frey *et al.*, 2008; Roach, 2011). SAM will bind to the iron–sulphur cluster [4Fe–4S] in any of the 2800 proteins which comprise the SAM superfamily, to form the [4Fe–4S]SAM 1⁺ complex which is cleaved to release methylthioadenosine and 5'-deoxyadenosine radical. The range and energy of the chemistry which the radical enzymes can perform is as astonishing as that performed by Vitamin B12, and they include biotin synthase, bacterial antibiotic synthesis and possibly synthesis of lipoic acid which is donated to the subunits of the pyruvate dehydrogenase complex acid in mammals (Grillo and Colombatto, 2007; Frey *et al.*, 2008; Roach, 2011). The generation of the 5'-deoxyadenosine radical is very endergonic (54 kJmol⁻¹) and partly explains why all three phosphate groups of adenosine triphosphate (ATP) are hydrolysed (50 kJmol⁻¹) during SAM synthesis (Roach, 2011). It also explains why the chemistry which is accomplished by radical SAM enzymes is so energetic. Most recently, it has been shown that mammalian viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible) is a radical SAM enzyme which might exert its antiviral action through disruption of membrane lipid rafts and prevention of viral release from infected cells. Viperin appears to inhibit farnesyl diphosphate synthase (FPPS), which is in the pathway of biosynthesis of isoprenoids, squalenes and sterols which are necessary to maintain membrane fluidity (Duschene and Broderick, 2010).

22.4.3 Methylation reactions

This is the most significant pathway of biological methylation of macromolecules such as RNA, DNA and protein, and also of cellular metabolites (Fig. 22.2). Histidine residues in muscle actin and myosin are methylated after synthesis of the protein to form 3-methylhistidine (Asatoor and Armstrong, 1967). Radiolabelled 3-methylhistidine is not incorporated into muscle protein *in vivo* but is quantitatively excreted, unchanged, in urine (Young *et al.*, 1972). It was thought that urinary 3-methylhistidine excretion would be a good biomarker for myofibrillar protein breakdown and thus muscle breakdown in growth and in wasting conditions (Nishizawa *et al.*, 1975, 1975) or in children suffering from protein–calorie

malnutrition or severe burns injury (Bilmazes *et al.*, 1978; Munro and Young, 1978; Nagabhushan and Narasinga Rao, 1978). However, analysis of incorporation rates of [^{14}C -methyl]methionine into muscle protein 3-methylhistidine or pulse-decay rates of urinary 3-methylhistidine after [^{14}C -methyl]methionine injection showed that skeletal muscle was not the main source of urinary 3-methylhistidine in the rat (Bates *et al.*, 1979; Millward *et al.*, 1980) and this is likely also to be true in humans (Rennie *et al.*, 1984).

SAM is also methyl donor for synthesis of symmetric and asymmetric dimethylarginine (ADMA) which are inhibitors of nitric oxide synthase. The ratio of plasma arginine/ADMA is low in patients with primary cardiovascular disease or following renal transplantation, and this is also related to a low methylation status, that is SAM/SAH ratios are lower than normal (Boger, 2008; Andrade *et al.*, 2011).

Methylation of nascent RNA in the nucleus or nucleolus is a key step in the subsequent correct processing of the RNA so that mature transcripts are transferred to the cytoplasmic compartment (Nazar, 2004; Boisvert *et al.*, 2007). Pre-RNA sequences containing methylated nucleosides are conserved in cytoplasmic RNA (Vaughan Jr *et al.*, 1967; Wagner *et al.*, 1967). In methionine-limited media, nucleolar precursor rRNA processing was slower than normal (Wolf and Schlessinger, 1977), but methionine deprivation led to formation of abnormal nucleolar precursor RNA in cultured HeLa cells which was 'wasted' before transport out of the nucleolus (Vaughan *et al.*, 1967). Modified nucleosides (109 different types) are produced post-translationally in all of the major RNA species whose sequence and methylation sites are described in the RNA Modification Database (Cantara *et al.*, 2011). The most diverse nucleoside modifications are purine/pyrimidine ring methylations, but the quantitatively most important are methylations on the 2'OH position on ribose, notably in rRNA (Maden and Hughes, 1997) but less so in mRNA (Desrosiers *et al.*, 1975). The rarest modifications are double modifications on sugar and base ring as in the case of 2'-O-methylpseudouridine (Maden and Hughes, 1997) or 2'-O-methyl-5-formylcytidine (Pais de Barros *et al.*, 1996). The commonest modification is not a methylation but isomerization of uridine to pseudouridine (Ψ or Pseu) which occurs at about the same concentration as the 2'-O-methyl nucleosides in rRNA (Hughes *et al.*, 1976). The excretion of these modified nucleosides as nucleosides or nucleobases has been widely reported as biomarkers for RNA and protein metabolism (Grimble *et al.*, 2000; Maïoral *et al.*, 2001) and cancer (Henneges *et al.*, 2009).

DNA methylation occurs most frequently at repetitive sites with high frequencies of the sequence motif CpG close to gene promoter regions. These CpG clusters or CpG islands are the site of methylation which tends to silence the promoter, and this may have profound effects on cell function, from silencing of tumour suppressor genes to silencing of genes responsible for maintenance of stem cell pluripotency (Illingworth and Bird, 2009; De Carvalho *et al.*, 2010). Two examples of the importance of methylation status are hypotheses which relate global DNA hypomethylation to the expression of the autoimmune disease Lupus erythematosus (Szyf, 2010) and to oncogene expression through promoter demethylation. This hypothesis attempts to explain the link between excessive alcohol intake (which interferes with SAM metabolism) and increased risk of colon cancer (Purohit *et al.*,

2005). In both cases, the proposed therapies involve improvements in methyl status through dietary means such as SAM or folic acid supplements (Fig. 22.2).

Finally, the majority of methylation flux (90%) is directed into the synthesis of sarcosine (glycine methylation) or creatine (guanidinoacetate methylation), both of which are estimated to consume 45% of the total (Mudd *et al.*, 2007). To put this into perspective, the estimated labile methyl flux in a 70 kg man would be 14–17 mmol/day against a dietary intake of methyl-containing compounds such as betaine (methyl intake ca. 2 mmol/day) and choline (recommended average methyl intake ca. 12 mmol/day) (Mudd *et al.*, 2007; Zeisel and da Costa, 2009). It is likely that most Americans consume less than the intake of choline recommended by the Institute of Medicine, Food and Nutrition Board and so methyl status is probably more precarious than was previously thought, a situation which can only be partly alleviated by folic acid supplementation since this compound is a methyl carrier not a net contributor to total methyl supply (Zeisel and da Costa, 2009).

22.5 Sulphur amino acid requirements following injury or infection

The focus here is on the quantitative significance of pathways of sulphur amino acid metabolism because they impinge on important cellular processes such as maintenance of intracellular redox status (GSH/GSSG), apoptosis (GSH, SAM), the inflammatory response (cysteine, GSH, SAM), cellular growth (methionine, SAM) or enzymology (radical SAM). In this respect, sulphur amino acid requirements have been reasonably well defined, as described above. However, when considering patients who are chronically or critically ill, the sulphur amino acid requirements are less clear and there may be two reasons for increased needs. In the case of ‘classical’ injury, Cuthbertson showed that urinary sulphate excretion increased three to eight days after injury at the peak of the catabolic response, a metabolic phenomenon which represents loss of muscle protein and increased cysteine and methionine oxidation (Cuthbertson, 1931). More recent studies in weight-stable patients with HIV infection have shown the same phenomenon (Breitkreutz *et al.*, 2000), but this could not have arisen from muscle loss. It has been argued that simple injury requires replacement of essential and conditionally-essential amino acids, even though the amounts remain to be defined (Furst and Stehle, 2004). However, during the early part of the injury response, which is driven by the innate and adaptive immune system, there may be a specific requirement for increased sulphur amino acid intake (Grimble and Grimble, 1998; Grimble, 2006).

22.6 Is supplementation with methionine and cysteine alone the best way forward?

Methionine and cysteine are toxic when given in excess (Baker, 2006) and a 1992 report attempted to provide guidance on this issue because manufacturing

advances meant that most amino acids had become widely available in high purity for use by farmers (to supplement animal feedstock), by athletes (to enhance performance), by clinicians (for artificial nutrition) and for the public to use as supplements (Anderson and Raiten, 1992). In the clinical arena, acute toxicity has been the main criterion because the conditions being treated are often life-threatening and toxicity has to be balanced against efficacy.

The commonest cysteine precursors are NAC and its other N-alkyl derivatives (Hsu *et al.*, 2004; Atkuri *et al.*, 2007; Grimble, 2007a) or L-2-oxothiazolidine-4-carboxylate (also known as OTZ, Thioproline) which is an analogue of oxoproline, the form in which glutamine is released from GSH. OTZ is converted to cysteine by oxoprolinase (Fukagawa, 2006). Glutathione analogues such as GSH monoethylester, S-acetylglutathione or N-butanoylglutathione have also been considered as antiviral agents because they have better stability and bioavailability than oral GSH (Fraternale *et al.*, 2009).

22.7 Paracetamol (acetaminophen) overdose as a paradigm for sulphur amino acid supplementation

Paracetamol is widely used as an over-the-counter analgesic and is mostly metabolized in the liver by conjugation with sulphate or glucuronide. However, some is converted by the cytochrome P-450 system to the toxic metabolite, N-acetyl-p-benzoquinoneimine. This is conjugated with glutathione and, upon removal of the glycine and glutamine moieties, is excreted as mercaptopuric acid and cysteine adducts (Black, 1984; McClain *et al.*, 1999). Overdose will overwhelm the capacity for glutathionylation and excess toxic metabolite binds to intracellular proteins, especially mitochondrial membrane proteins, causing mitochondrial dysfunction and endoplasmic reticulum stress. An important facet of this process is that increased ROS formation results in DNA damage and ultimately activation of the cellular necrosis (not apoptosis) pathway (Jaeschke *et al.*, 2011). In animal models of paracetamol overdose, the initial catastrophic depletion of GSH is very rapid (one hour) and correlates with the clinical window (15 hours) for effective treatment with GSH replenishing compounds (e.g. NAC) which will prevent liver failure. There is no doubt that cytokines are involved in the elaboration of this type of liver injury, but they are much less important than the early oxidant stress arising from GSH depletion (Bajt *et al.*, 2004). The importance of these findings cannot be overstated because they have provided the basis for treatment of liver disease with GSH precursors which restore the intracellular redox potential in conditions as diverse as kwashiorkor (Krawinkel, 2003), viral hepatitis (Barbaro *et al.*, 1996) and alcoholic or non-alcoholic fatty liver disease (Baumgardner *et al.*, 2008; Ronis *et al.*, 2011).

A second factor which is less obvious is that restoration of the intracellular SAM pool is also hepatoprotective against paracetamol toxicity in mice (Bray *et al.*, 1992; Terneus *et al.*, 2008) and in dogs (Wallace *et al.*, 2002). There is a considerable literature on the clinical use of SAM in alcoholic and viral liver

disease (e.g. see (Feld *et al.*, 2011; Rambaldi and Gluud, 2009)). At present, there are no human data to show efficacy in paracetamol poisoning and the reason for slow progress is possibly that there is already a good alternative in the form of NAC which is well tolerated and effective (Aitio, 2006; Atkuri *et al.*, 2007). Oral SAM is reasonably well tolerated (Rosenbaum *et al.*, 1990) and is now becoming available in high-purity formulations with good stability (Morana *et al.*, 2000), and intravenous SAM delivered as liposome preparations loaded with S-adenosyl methionine 1,4-butanedisulphonate is more hepatoprotective against paracetamol poisoning in mice than the native compound (Wagner *et al.*, 2010). A comparison of SAM and NAC in mice poisoned with paracetamol, showed equal effectiveness in preventing liver failure and death, and salvaged liver GSH pools equally, but SAM prevented the appearance of focal necrosis around the central hepatic vein and also reduced the amount of 4-hydroxynonenal-protein adducts (arising from membrane lipid peroxidation by ROS) more than NAC (Terneus *et al.*, 2008).

22.8 S-adenosylmethionine and infection

22.8.1 Viral hepatitis

Hepatitis C viral (HCV) infection significantly reduces intracellular hepatic GSH, depending on the viral genotype, and is related most closely to the appearance of irregular, dysfunctional mitochondria (Barbaro *et al.*, 1999a). The degree of GSH depletion relates to the histological severity of liver damage, but this is complicated by the common incidence of co-infection with HIV (Barbaro *et al.*, 1999b). The most recent trial of SAM as a co-adjuvant therapy in non-responding patients treated with pegylated interferon and ribovirin is very encouraging. The rate of viral decline was increased and nearly a half of patients had no detectable HCV RNA 24 weeks after SAM therapy (Feld, *et al.*, 2011). One explanation for this effect was that SAM increased expression of the interferon-stimulated genes (ISG) which are an antiviral defence. The most interesting finding was that SAM treatment *in vivo* and *in vitro* led to increased expression of the signal transducer and activator of transcription (STAT1) which is a promoter of ISG. There was increased methylation of arginine 31 on STAT1, which prevents inhibition by other factors (Duong *et al.*, 2006; Feld *et al.*, 2011). These findings imply that improvement in redox status and methyl status were of benefit, but the reasons are unclear. Increased intracellular SAM (30–50 $\mu\text{mol/L}$) may increase methylation rates, but the K_m for protein arginine methyltransferases is in the low μmolar range and should always be saturated for SAM as substrate (Obianyo *et al.*, 2007, 2008). However, SAH is a potent inhibitor (Feng *et al.*, 2011) and the increased SAH–SAM ratio in infected liver cells may be inhibitory, as discussed elsewhere (Kharbanda, 2009).

22.8.2 HIV infection

Before antiretroviral therapy was routinely used, many patients with HIV infection developed poor nutritional status early in the course of infection (Bogden *et*

et al., 2000) and, specifically, reduced GSH status was shown to be a marker of poor prognosis. It was known that NAC and OTZ were able to block TNF- α -induced HIV replication *in vitro* (Roederer *et al.*, 1990; Raju *et al.*, 1994) and, in addition, patients with active HIV infection catabolized large amounts of cysteine (Breitkreutz *et al.*, 2000). The reason for this lies in the reduction of intracellular GSH and oxidized glutathione (GSSG) concentrations mediated partly by net export of GSSG as a means of maintaining a low intracellular redox potential. Several clinical trials of agents which replenish intracellular GSH have been performed. Alpha-lipoic acid (ALA), an endogenously synthesized naturally occurring disulphide, has been shown to improve reactivity of T-lymphocytes to mitogens (Jariwalla *et al.*, 2008). In contrast, supplementation with NAC (Treitinger *et al.*, 2004) or whey protein supplement with high sulphur amino acid content (Micke *et al.*, 2001) or with OTZ (Barditch-Crovo *et al.*, 1998) had no effect on lymphocyte reactivity even though all agents increased blood GSH concentrations.

22.9 Sulphur amino acids and their effect on illnesses

22.9.1 Chronic inflammatory conditions

Early studies of SAM to treat osteoarthritis showed great promise (Bradley *et al.*, 1994; Najm *et al.*, 2004) and there have been numerous explanations for the mechanisms involved which include (as with the case of viral infections): increased glutathione; reduced cytokine release; stimulation of cartilage synthesis; and alteration of gene expression through methylation of DNA or of transcription factors (Hosea Blewett, 2008). Three meta-analyses have reported that oral SAM has no effect (Witte *et al.*, 2002), has limited and clinically relevant effects on disease and pain (Rutjes *et al.*, 2009) and significant effects equivalent to non-steroidal anti-inflammatory drugs (Soeken *et al.*, 2002). This divergence of analysis is partly due the small number of adequately powered clinical trials and varying inclusion criteria for the meta-analyses.

Fibromyalgia is defined as '*a condition marked by chronic widespread pain and multiple symptoms*' which relate to disorders of sleep, mood and irritable bowel syndrome, for example (Clauw, 2009). The most effective treatments for this distressing condition are antidepressants or other centrally acting agents, but two early trials showed some benefit from oral SAM by an, as yet, unknown mechanism (Tavoni *et al.*, 1987; Jacobsen *et al.*, 1991). The most recent meta-analyses concluded that SAM therapy had '*the most potential for further research*' (Porter *et al.*, 2010) or '*insufficient evidence*' to support it (De Silva *et al.*, 2010). The most interesting feature of this topic is that SAM has been shown to have some effects in treatment of psychological depression (Williams *et al.*, 2005; Freeman *et al.*, 2010), but the mechanisms remain speculative and centre on modulation of intracellular neural Hcy/SAH by SAM treatment (Fuh, 2010).

22.9.2 Critical illness

Glutathione concentrations were found to be significantly reduced in muscle and plasma, but not whole blood of patients who were mechanically ventilated, post-surgical, suffering from trauma, cardiogenic shock or had multisystem organ failure (MSOF) (Hammarqvist *et al.*, 1997; Luo *et al.*, 1997; Flaring *et al.*, 2005). This matches findings in skeletal muscle of patients in cardiogenic shock (Corbucci *et al.*, 1985) and of animals subject to circulatory shock and trauma in which muscle mitochondrial dysfunction was also observed. This is a general phenomenon in critical illness and serves to improve survival, even in MSOF for reasons outside the scope of this chapter (Singer, 2007). However, the ability to maintain antioxidant defences following serious trauma is important. Markers of oxidative damage increase in all ICU patients but normalize in survivors and continue rising in non-survivors (Mishra *et al.*, 2005). This implies a failure of antioxidant defences and may be of key importance in treating critical illness. Whilst the inflammatory response to injury is usually a self-limiting process in which endogenous nutrients (e.g. glutamine, cysteine) are mobilized to play their part (Grimble, 2007b), a rapid progression may take place from acute injury to systemic inflammatory response syndrome (SIRS) and sepsis and, finally, MSOF which carries a high risk of death (Singer, 2007; Castellheim *et al.*, 2009). In this situation, sulphur amino acid therapy has been proposed as a means of preserving antioxidant defences and 'blunting' this inflammatory cascade.

More recent studies of this approach have generally given negative results. NAC therapy has been shown to restore intracellular glutathione (Paterson *et al.*, 2003), but this should be considered in the light of a more detailed investigation of blood peroxides and antioxidant enzyme defences in patients with peritonitis which failed to confirm that disordered antioxidant defences were prognostic (Kumar *et al.*, 2007). One clinical trial has shown that supplementation with two other antioxidants related to glutathione, α -tocopherol and ascorbate, reduced the risk of progression to MSOF by 57% (Nathens *et al.*, 2002), whereas a large trial of glutamine and selenium supplementation showed no effect (Andrews *et al.*, 2011). The results of the 1200 patient multicentre REDOXS Study (REDucing Deaths due to OXidative Stress) showed that early provision of glutamine or antioxidants did not improve clinical outcomes, and glutamine was associated with an increase in mortality among critically ill patients with multiorgan failure (Heyland *et al.*, 2013). It can be concluded that restoration of antioxidant defences is a part of the picture but by no means the main factor.

22.9.3 Manipulation of sulphur amino acid metabolism in cancer

The two approaches which have been tried seem contradictory and rely on (i) improvement of GSH metabolism in HCV infection or alcoholic liver disease, in order to prevent progression to liver cancer, and (ii) impairment of methionine metabolism, in order to augment chemotherapy.

The model of treatment with carcinogens (e.g. diethylnitrosamine) has been used to study liver cancer development because it reproducibly shows a progres-

sion from preneoplastic lesions to cancer. Early studies suggested that SAM injections for an extended period not only replenished the intracellular SAM pool in liver but also markedly retarded the development of lesions (Pascale *et al.*, 1991, 1992). SAM treatment increases GSH pools but, by itself, is capable of exerting powerful inhibitory effects on the activation of NF κ B and expression of TNF- α (Arias-Diaz *et al.*, 1996; Garcia-Roman *et al.*, 2008). The effect is interesting because SAM is also protective against TNF- α -induced liver injury following lipopolysaccharide injection. The effect is not mediated by increased GSH because SAM was unable to replenish this pool whereas NAC could but was not hepatoprotective (Watson *et al.*, 1999). One explanation for these differential effects is that increases in SAM stimulate hepatocyte cancer cell apoptosis but suppress it in normal hepatocytes (Mato and Lu, 2011). Support for this plausible explanation came from the finding that *in vivo* over-expression of methionine adenosyl transferase (MAT) in liver cells rendered them resistant to transformation because intracellular SAM synthesis was greatly increased (Li *et al.*, 2010); in contrast, over a half of MAT knockout mice spontaneously developed liver cancer (Martinez-Chantar *et al.*, 2002).

This is relevant to the association between liver cancer and heavy alcohol consumption which upregulates cytochrome P450 2E1-dependent ethanol metabolism and activation of procarcinogens by the same pathway (McKillop and Schrum, 2005). Animal models of chronic alcohol consumption show a predictable progression of fatty infiltration, lipid peroxidation and increased TNF- α and transforming growth factor β which is almost completely reversed by SAM treatment (Gong *et al.*, 2008). In addition, Toll-like receptor expression which initiates the pathway of stem cell progression to cancer is also inhibited by SAM therapy in animal models of alcohol- or carcinogen-induced liver disease (French *et al.*, 2010). The most recent meta-analysis of clinical trials for use of SAM in alcoholic liver disease has shown no benefit but did note that there are insufficient, adequately-powered trials to allow firm conclusions to be drawn (Rambaldi and Gluud, 2009). Finally, it is now clear that non-alcoholic fatty liver disease also involves a similar derangement in methionine metabolism and it is suggested that SAM may be hepatoprotective against development of steatohepatitis and cirrhosis (Kalhan *et al.*, 2011).

In one intriguing series of studies, Goseki and colleagues explored the effect of methionine-depletion (methionine-stress) on the effectiveness of chemotherapy. They hypothesized that deliberate reduction in GSH defences would sensitize tumour-bearing animals to the effect of chemotherapy; in other words, the agent would induce necrosis/apoptosis in tumour cells at an increased rate (Goseki and Endo, 1990). Rats bearing Yoshida sarcoma received total parenteral nutrition based on Rose's recommendations but lacking methionine and it was found that tumour growth was markedly retarded and survival increased (Goseiki *et al.*, 1991, 1992). *In vitro*, the same effect has been observed in pancreatic and neural tumour cell lines (Kokkinakis *et al.*, 2004, 2005). In the only human clinical trial to date, methionine-depletion and 5-fluorouracil (5FU) treatment before surgery for resection of gastric cancer showed marked regression of tumour sites examined by

histology, compared to complete total parenteral nutrition and 5FU (Goseki *et al.*, 1995a,b). The mechanisms behind the effect of methionine-stress are complex and involve inhibition of DNA methylation, activation of cell cycle check points and the inhibition of survival pathways (Kokkinakis, 2006).

22.10 Conclusion and future trends

There is no doubt that impairment of any part of the methionine cycle or its subsidiary pathways will lead to significant impairment of cellular processes and underlies the effectiveness of anticancer therapies which target 1-carbon metabolism (e.g. methotrexate). This review has explored a wide literature in order to see how modulation of sulphur amino acid metabolism might be useful in modifying the inflammatory response. Most of the treatments can be broadly classed as 'nutritional' in the sense that these are agents given in significant amounts orally or intravenously with other nutrients and are compounds which are generally found in foods that we eat in small amounts (except for OTZ). They are 'natural' (rather than 'pharmaceutical') and this has undoubtedly helped internet sales as 'alternative' therapies. This is unfortunate because self-medication escapes the rigours of clinical trials and drug surveillance; indeed the label 'natural' may be counterproductive and reduce the importance which clinicians place on a treatment. Two recent reviews of alternative therapies, for example, ranked SAM alongside St John's Wort, anthocyanidins and homeopathy as treatments for fibromyalgia and depression (De Silva *et al.*, 2010; Carpenter, 2011). These reviews were not 'grey' literature but a serious attempt to define the efficacy and effectiveness of these treatments when the evidence is quite feeble. It should not obscure the fact that alteration of intracellular GSH or SAM pools has profound effects on expression of pathways for cell survival, apoptosis and necrosis. SAM and NAC supplementation show great promise in treatment of liver disease arising from viral infection or alcohol abuse, because of their effects on apoptosis, mediated partly through GSH pools and through methylation-dependent pathways. In contrast, NAC is more problematic in the face of the three ugly sisters of critical illness: the cytokine storm, fluid and acid-base imbalance and reduced redox defences. The consensus for clinical ICU trials seems to be that maintenance of intracellular GSH is not a panacea for these patients.

One can conclude that sulphur amino acid therapy is all about timing. One intriguing study epitomizes this approach in relation to HIV infection. '*Shock and kill*' strategies are designed to activate the virus then kill the infected cells through natural means. Inhibition of GSH synthesis by buthionine sulfoxide (BSO) increases oxidative stress in HIV-infected cells leading to HIV-1 activation (see above). *In vitro*, it provides a window for cell-killing effectiveness of the new class of histone deacetylase inhibitor targeted at infected cells (Savarino *et al.*, 2009). Both drugs could therefore be used together at subtoxic levels but with improved effectiveness. In this sense, it is the exact counterpart of the methionine-depletion studies in cancer and the inverse image of NAC treatment of paracetamol overdose.

Sulphur amino acid metabolism and its manipulation is clearly moving towards an interesting therapeutic future.

22.11 References

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Part IV

Nutrition, immunity and inflammation

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23

Adverse immune reactions to foods

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Abstract: The increasing prevalence of immune-mediated adverse reactions to food represents a significant personal and societal burden. These reactions can manifest as a range of debilitating and life-threatening conditions. This chapter describes the main immune-mediated adverse reactions to food including IgE-mediated and non-IgE-mediated food allergies and coeliac disease. It considers the prevalence, immune mechanisms, presentation and management of these disorders along with novel therapeutic strategies being developed. The chapter discusses the breadth of research activity into all aspects of these reactions, from the mechanism and natural history, to diagnosis and new treatments. This research is increasing our understanding and leading to novel management strategies.

Key words: food allergy, food-induced anaphylaxis, coeliac (celiac) disease, eosinophilic, food-protein induced enteropathy.

23.1 Introduction

The term ‘adverse reaction to food’ encompasses a diverse array of pathological responses to a wide variety of foods. Symptoms range from unpleasant (rashes, abdominal discomfort) to severe or even fatal (anaphylaxis, organ failure). Due to the diversity of the causative mechanisms of these responses it can be difficult to categorise them. A simple and useful division is to define adverse reactions to food as either intrinsic to the host organism or extrinsic, that is dictated by a characteristic of the food (Fig. 23.1). Extrinsic factors would encompass all food poisoning whether a result of contamination of that foodstuff with a toxic agent (mercury, arsenic, dioxins) or pathogenic organisms (bacteria, viruses and fungi). Sometimes the toxic contaminant is the product of organisms which in themselves may not harm the host, but produce toxic compounds which are damaging to the host. Such

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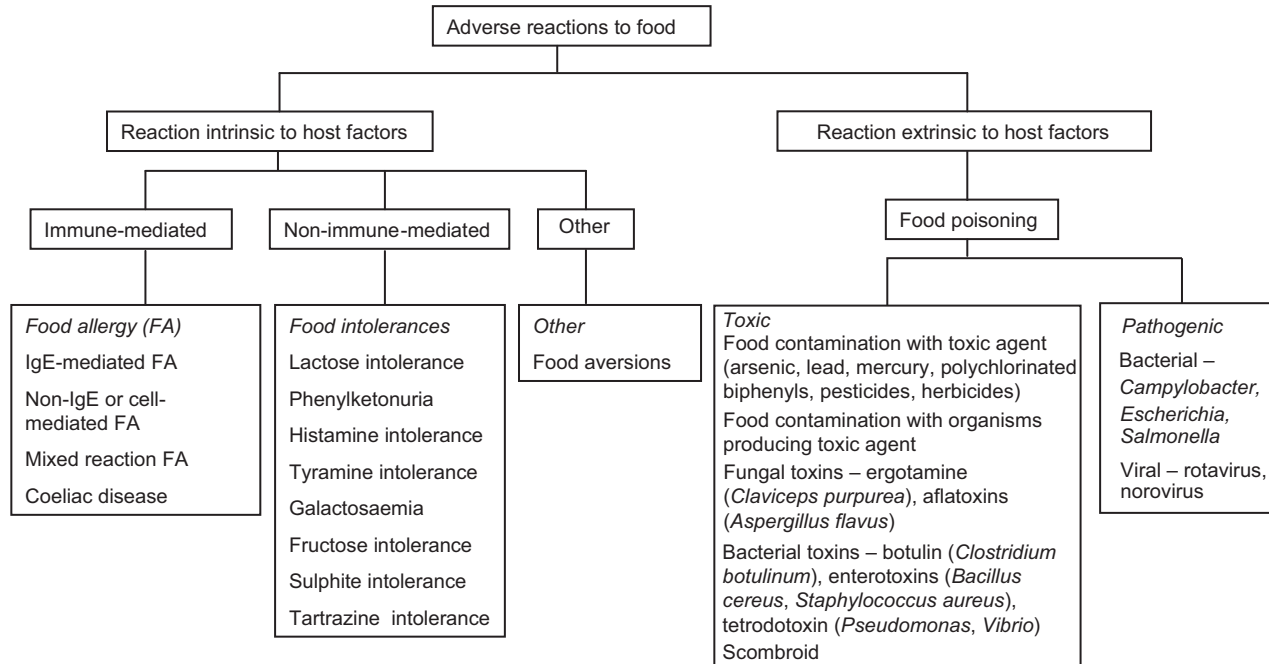


Fig. 23.1 Adverse reactions to food may be caused by factors which are intrinsic to the host or those dictated by factors relating to the foodstuff. Host factors include the inability to metabolise particular dietary components (sugars, amino acids) often caused by particular enzyme insufficiencies. These reactions are commonly termed food intolerances. Adverse reactions caused by immune mechanisms intrinsic to the host are commonly termed food allergies. These may be mediated by the antibody type which is associated with allergy, immunoglobulin E (IgE), by another mechanism not involving IgE, or sometimes by both IgE and other immune mechanisms (mixed reaction). Coeliac disease is caused by an adverse immune response to a food and may be viewed as a non-IgE mediated reaction. However, it is distinguished from the other food allergies by the involvement of an autoimmune reaction (to host 'self' antigens) and the restricted types of allergen which drive the reaction (gluten from wheat or closely related cereal grains).

is the case for the fungus ergotamine (*Claviceps purpurea*). This fungus infects rye and other cereals and produces the alkaloid toxin, ergotamine. This is a neurotoxin and ingestion results in seizures, spasms and convulsions and is frequently fatal. In the middle ages, these symptoms were termed St Anthony's fire. There are still occasional ergotamine poisoning outbreaks, particularly in the developing world such as that seen in Ethiopia in 2001.

Adverse reactions to food dictated by intrinsic factors include those caused by enzyme defects or insufficiencies resulting in an impaired ability to adequately metabolise particular compounds (e.g. phenylalanine, histamine, tyramine, lactose, fructose, galactose). The inability of the body to metabolise the compound results in an increasing concentration of the compound or by-products causing a pathological response. In the case of phenylketonuria, resulting from the inability to metabolise phenylalanine, the severity of the response has resulted in the adoption of a national screening programme, testing all infants within a few days of birth for the concentration of phenylalanine in the blood.

Lactose intolerance is perhaps the most prevalent adverse reaction to food, affecting approximately 70% of the world population. It is caused by an insufficiency of the enzyme lactase, resulting in an impaired ability of the body to metabolise the milk sugar, lactose. The passage of the lactose into the colon causes symptoms of distension, pain, increased flatulence and diarrhoea. For a very small proportion of the lactose-intolerant population, the defect is a result of a rare genetic disorder resulting in a severe lactase insufficiency present from birth. These patients need to be diagnosed and managed with lactose-free nutrition from a very early age to prevent longer term effects on their growth and development. The majority of lactose-intolerant individuals are born with sufficient lactase production to metabolise lactose in breastmilk. The lactase activity reduces in infancy and finally is almost completely lost by adulthood. This transient lactase production is the developmental norm for humans but has been altered in some populations to retain lactase production throughout life. It is thought that this has been brought about by selection pressure on the population. It has been speculated that this pressure may have been the result of living in more northern latitudes where sunshine exposure would be reduced compared with tropical or subtropical regions. The lack of sunshine would confer a reduced vitamin D status and resultant impaired bone mineralisation in these populations. The consumption of milk would provide a dietary source of calcium and vitamin D. The gene sequence changes to the gene controlling lactase production, and conferring lifelong production of the enzyme, appeared in the human genome at about the same time as animal husbandry became prevalent in the populations, about 10 000 years ago.

This chapter will focus on adverse reactions to food dictated by intrinsic factors of an immune nature. These include food allergies which may be caused by mechanisms mediated by the allergic antibody, immunoglobulin (Ig) E, or by cell-mediated immune responses (with or without the activity of IgE). In addition, there are adverse immune responses to food for which the pathology, although driven by a food antigen, is the result of an autoimmune reaction (coeliac disease, CD).

The appearance of adverse immune responses to food may appear to result from the development of a 'new' reaction to a food antigen. In reality, it is perhaps more accurate to view it as the failure of the body to maintain the stance of immunologically 'ignoring' (i.e. tolerating) the vast array of non-self food-derived antigens, whilst still responding to pathological agents within the gut. This ability of the immune system to discriminate between harmful and innocuous foreign or non-self substances is the result of complex interactions between the cells which sample the gut contents and 'show' it to the immune system (antigen presenting cellsm, APC) and the immune cells. The signals which the immune cell receives during the encounter with the antigen presenting cell will dictate whether the immune cell will be activated or remain quiescent. This immunological quiescence in response to selected substances (antigens) is termed immune 'tolerance'. It is the loss of this state of tolerance which results in an adverse immune response to a food.

23.2 Food allergy classification: IgE-mediated, non-IgE-mediated, and mixed IgE- and non-IgE-mediated food allergy

The term 'food allergy' has been defined as 'an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food' in the Guidelines for the Diagnosis and Management of Food Allergy in the United States (Boyce *et al.*, 2010). Foods commonly causing food allergy include cow's milk, hen's egg, wheat, peanuts, tree nuts, soy, fish and shellfish. In the USA the Food and Drug Administration has identified a list of the top food sources of allergens as milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, soybeans and wheat. However, more than 170 different foods have been reported to cause food allergy.

The true prevalence of food allergy is difficult to establish, but large-scale studies such as the EuroPrevall have been designed to give a better insight into patterns and prevalence of food allergy. Existing figures of self-reported food allergy, reviewed from 51 publications from a number of European and other countries, suggested that prevalence of food allergy to any food ranges between 3 and 35% (Rona *et al.*, 2007). Breaking these figures down to the individual foods, self-reported food allergy to milk ranged from 1.2–17%, for egg 0.2–7%, for peanut 0–2%, for fish 0–2% and for shellfish 0–10%. In the same review, the limited data from studies using a positive response to a food challenge as a diagnosis for food allergy gave a prevalence of 1–10.8% to any food. In the USA, prevalence for food allergy has been estimated at up to 8% of children below three years of age and 3.9% in children < 18 years (Sampson, 1999; Branum and Lukacs, 2009). The prevalence of food allergy appears to have increased over the last few decades. In the USA, a 2009 report suggested that from 1997 to 2007 there was an 18% increase in paediatric food allergy (Branum and Lukacs, 2009). In the UK, hospital admissions for food allergy showed a five-fold increase from 1990/1 to 2003/4 (Gupta *et al.*, 2007). The greatest increase in hospital admission rates for food allergy (seven-fold) was seen in the paediatric population (Gupta *et al.*,

IgE-mediated immune mechanisms	Non-IgE-mediated immune mechanisms	
Immediate, type I hypersensitivity	Eosinophilic oesophagitis	Food protein-induced (allergic) proctocolitis
	Eosinophilic gastroenteritis	Food protein-induced enterocolitis syndrome
		Food protein-induced enteropathy
		Coeliac disease (gluten-sensitive enteropathy)

Fig. 23.2 Adverse reactions to foods caused by immune mechanisms are often classed as IgE- or non-IgE-mediated reactions. IgE-mediated reactions are those which are immediate, type I hypersensitivity immune responses to foods. Non-IgE-mediated reactions to food include a number of distinct syndromes including food protein-induced (allergic) proctocolitis, food protein-induced enterocolitis syndrome and food protein-induced enteropathy. Coeliac disease is distinguished from the other non-IgE-mediated reactions to food by the presence of an autoimmune reaction (to host ‘self’ antigens) and restricted causative allergen specificity (gluten or gluten-related proteins from wheat, rye or barley). There are also adverse reactions caused by immune mechanisms involving IgE-type mechanisms and other mechanisms, such as eosinophilic oesophagitis and eosinophilic gastroenteritis. These are termed IgE- and non-IgE-mediated reactions or mixed reactions. There is often concurrent allergy with these syndromes and the presence of allergen-specific IgE. However, the mechanism is not a type I hypersensitivity immune response to foods and involves other immune responses which are not yet fully elucidated.

2007). These estimated prevalence figures and time trends indicate why food allergy is an increasingly significant burden on society.

The categorisation of types of food allergy may be tackled in a number of ways. Some choose to divide food allergies up in terms of the initiating allergen, e.g. cow’s milk allergy or peanut allergy. However, reactions to a single food source may be caused by different immune responses. In this chapter, food allergies will be defined by the nature of the immune response causing the pathology. Broadly, food allergic responses may be divided into those for which pathology is mediated by the presence the antibody immunoglobulin (Ig) E, termed ‘IgE-mediated’ or those not necessarily requiring the presence of antigen-specific IgE, ‘non-IgE-mediated’. The non-IgE-mediated food allergy group includes a number of different conditions (Fig. 23.2), e.g. food protein-induced allergic proctocolitis and food protein-induced enterocolitis. There are also a number of food allergic responses which involve both IgE-mediated and non-IgE-mediated mechanisms, e.g. eosinophilic oesophagitis, eosinophilic gastroenteritis.

23.3 IgE-mediated food allergy

Sometimes termed immediate, or type I hypersensitivity, symptoms are typically apparent with minutes to hours of ingestion of the food allergen. The response may vary in severity from skin rashes, mouth tingling or swelling and gastrointestinal discomfort to severe reactions of respiratory distress, rapid and significant fall in blood pressure, loss of consciousness and death. The rapid onset after ingestion of

antigen and possible severity of the response are two reasons why IgE-mediated allergy was the earliest and best characterised food allergic reaction.

23.3.1 Immune mechanism

Primary exposure and sensitisation

The gastrointestinal tract is daily subjected to large quantities of a vast array of antigens. For the most part the gut-associated immune system is able to sample these antigens and distinguish between pathogens and innocuous antigens as they pass through the gastrointestinal tract. However, in some individuals an aberrant immune response is generated to food antigens resulting in the production of specific IgE. This immune response is initiated when antigens are sampled by APCs in the gut mucosa. These APCs process the food antigens and present fragments (epitopes) of the antigen to naïve T helper (Th) cells. Due to the types of mediators present and cell-to-cell signals encountered by the naïve Th cell at the time of priming, the Th cell is differentiated into a Th2 cell. The distinction of Th cells into Th1- or Th2-type is made by the pattern of cytokines which they produce. These in turn dictate the nature of the immune response which the primed Th cell will drive (Fig. 23.3). Th1 cells are characterised by the production of interleukin (IL)-2 and interferon gamma (IFN- γ) and are involved in antibacterial, antiviral and antitumour responses. Th2 cells are characterised by the production of interleukin IL-4, -5 and -13. IL-5 promotes eosinophil proliferation and prolonged survival. IL-4 causes B cell activation and proliferation and drives immunoglobulin class switching in the B cell resulting in IgE production. Similarly, IL-13 acts upon B cells to promote IgE production. This type of protective immunity is produced in response to parasitic infections (e.g. helminth) with the development of eosinophilia and high titres of antigen-specific IgE augmenting clearance of the parasites from the host. Conversely, the same mechanism, when developed in response to food antigens, is the first stage in developing food allergy. However, although the production of food antigen-specific IgE is necessary for IgE-mediated food allergy, it is not sufficient. Food-specific IgE can be detected frequently in individuals who do not suffer from any apparent adverse reactions when exposed to that food.

Secondary exposure

Food allergen-specific IgE binds to mast cells and basophils via high-affinity IgE receptors (Fc ϵ RI). Mast cells are present in most tissues and are found in the gut mucosa, skin, respiratory tract and lining the blood vessels. When this cell-bound IgE encounters the food antigen, the antigen binds, cross-linking the IgE molecules on the mast cells and causing activation of the mast cell. Mast cells are granulocytes and contain granules which are packed with preformed mediators such as histamine, proteoglycans and proteases. Once the surface IgE is cross-linked there is a rapid influx of Ca²⁺ into the mast cell, causing rapid degranulation and releasing the inflammatory mediators into the surrounding tissues. This causes

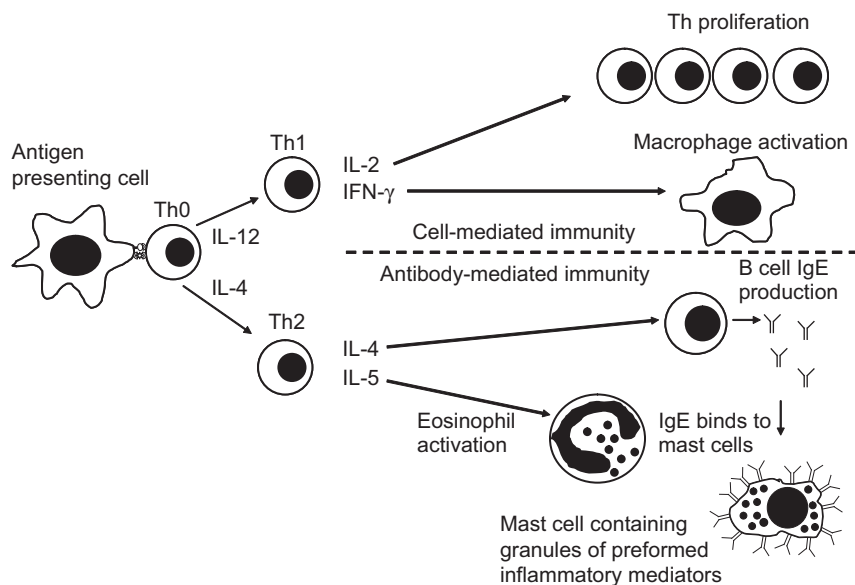


Fig. 23.3 Food allergy involves a memory or adaptive-type immune response, i.e. every time the food is ingested, specific T cells recognise the food antigen and react to it. This is initiated when an antigen presenting cell (such as a dendritic cell) takes up the food antigen and presents it to naïve T cells until it finds a match, i.e. a naïve T cell which recognises the antigen. During this presentation the surrounding cytokines in the environment and other receptor-based signals provided to the naïve T will dictate the kind of T helper (Th) cell it will become. IgE-mediated allergic reactions are orchestrated by antigen-specific Th2 cells. Non-IgE-mediated reactions will be a Th1 cell-driven reaction.

the very rapid response seen in IgE-mediated allergy. Histamine has a number of effects on the cardiovascular system such as increasing vasodilatation, vascular permeability and cardiac tachycardia and arrhythmia; these can cause flushing, oedema, reduced blood pressure, loss of consciousness, death. Effects on the respiratory system such as smooth muscle contraction and increased mucus secretion result in respiratory distress and, coupled with the effects on vessels (increased dilatation and permeability), can lead to congestion of the nose and sneezing. The increased smooth muscle contraction can cause cramping, vomiting and diarrhoea. Histamine also has effects on the central nervous system causing headache and nausea. Proteases (tryptase, chymase) also cause bronchial hyper-responsiveness and mucus over secretion. Preformed cytokines are also released (IL-5, IL-8) and these act as chemoattractants for other inflammatory cells (eosinophils, neutrophils, basophils). This is followed by the mast cell synthesis of newly formed mediators, particularly the prostaglandins and leukotrienes synthesised from arachidonic acid. When released, these mediators induce bronchoconstriction, oedema and mucus hyper-secretion and are the cause of the late phase of the IgE-mediated response (around six hours after the initial response).

23.3.2 Presentation

The symptoms of IgE-mediated food allergy may manifest as reactions in the skin, gastrointestinal tract, respiratory system or cardiovascular system. Symptoms of the skin include rapid responses (hives, angioedema) or, more rarely, chronic manifestations (urticaria, atopic dermatitis). Respiratory symptoms alone are rarely seen in response to food in IgE-mediated food allergy but normally seen in conjunction with other effects, frequently in anaphylactic responses (see below). The induced bronchial constriction and mucus secretion can cause rhinitis, wheeze, coughing and respiratory distress. Gastrointestinal symptoms usually manifest as acute and severe vomiting but may include abdominal cramping and diarrhoea. Anaphylaxis is a response in which a number of organs or systems are affected rapidly and simultaneously in an IgE-mediated allergic reaction. This is often very severe or even fatal. The World Health Organisation defines anaphylaxis as a severe, life-threatening generalised or systemic hypersensitivity reaction. However, as the term 'life-threatening' may be differentially interpreted, new and more defined criteria were set out by a report from the Second National Institute of Allergy and Infectious Disease/ Food Allergy and Anaphylaxis Network symposium (Sampson *et al.*, 2006). This concluded that anaphylaxis is highly likely when any one of the following three criteria are fulfilled:

1. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g., generalised hives, pruritis or flushing, swollen lips–tongue–uvula) *and at least one of the following*:
 - (a) Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced peak expiratory flow, hypoxemia)
 - (b) Reduced blood pressure or associated symptoms of end-organ dysfunction (e.g., hypotonia [collapse], syncope, incontinence)
2. Two or more of the following that occur rapidly after exposure to a *likely* allergen for that patient (minutes to several hours):
 - (a) Involvement of the skin-mucosal tissue (e.g., generalised hives, itch-flush, swollen lips-tongue-uvula)
 - (b) Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced peak expiratory flow, hypoxemia)
 - (c) Reduced blood pressure or associated symptoms (e.g., hypotonia [collapse], syncope, incontinence)
 - (d) Persistent gastrointestinal symptoms (e.g., crampy abdominal pain, vomiting)
3. Reduced blood pressure after exposure to a *known* allergen for that patient (minutes to several hours):
 - (a) Infants and children: low systolic blood pressure (age specific) or greater than 30% decrease in systolic blood pressure
 - (b) Adults: systolic blood pressure of less than 90 mmHg or greater than 30% decrease from that person's baseline

Anaphylaxis may be caused by a number of triggers with food allergens being the most common cause. Food-induced anaphylaxis accounts for about five to seven deaths per year in the UK and been recorded for individuals aged from five months to 85 years (Pumphrey and Gowland, 2007). The most common causative allergens were peanut, tree nut and milk. Similarly, in the USA, reports of fatal anaphylactic responses to food list peanut, tree nut and milk as the most frequent triggers (Bock *et al.*, 2007).

In addition, there is a more recently described form of anaphylaxis in which the reaction only occurs if the food is ingested following an episode of exercise, termed food-associated exercise-induced anaphylaxis. The exercise is presumed to alter gut absorption and/or digestion of the food allergen.

The symptoms of food allergy may also be induced by the cross-reactivity between an inhalent allergen and a food allergen. Oral allergy syndrome is also described as pollen-associated food allergy syndrome. It is IgE-mediated and manifests as a localised oral response (lips, mouth and throat) to foods such as fruits and vegetables (usually raw). Symptoms include itching of the lips, tongue, roof of the mouth and throat, with or without associated swelling, and it most commonly affects patients who are allergic to pollen. Such reactions involve cross-reactivity, e.g. that between the major birch tree pollen allergen Bet v 1 and the major allergen in apple Mal d 1, where there is a 63% homology between the sequences for the two allergens.

23.3.3 Diagnosis

The importance of a thorough clinical history of the patient, taking in as many relevant details as possible, cannot be over-emphasised. In the case of IgE-mediated food allergy, the acute onset of the response is normally a useful indicator of the diagnosis. The clinical history will normally indicate the causative food(s) allowing for more defined investigation. The detection of allergen-specific IgE is also a useful laboratory test to help confirm diagnosis and identify the triggering food. This may be done either by looking for specific IgE in a blood sample or by using the skin prick test. The skin prick test involves placing a small drop of allergen solution on the skin and gently breaking the skin below the drop. The allergen in the solution will make contact with any IgE bound to mast cells in the skin. If the IgE is specific for the allergen, the bound IgE will be cross-linked by the allergen causing mast cell degranulation, histamine release and a wheal and flare reaction (see Plates III and IV in the colour section between pages 516 and 517). This can be read 15–30 minutes after administration and compared with a positive (histamine solution) and negative (saline control solution) response. Both specific IgE titres and skin prick test are well established methods for determining allergen-specific IgE.

However, the presence of allergen-specific IgE, although necessary for an IgE-mediated response, is not sufficient. Many patients with specific IgE will not show any apparent adverse symptoms in response to exposure to that food. Therefore, detection of specific IgE by blood test or skin prick test is not diagnostic in itself.

However, there is evidence that the greater the IgE response detected (i.e. the higher the specific IgE titre or the larger the skin test wheal response) the greater is the predictive value of the specific IgE for causing food allergy symptoms. Furthermore, the absence of specific IgE by blood test or skin prick test may not preclude the diagnosis of an IgE-mediated response, as false negative results can be obtained. This may be due to the fact that the allergen used in the test does not bear the epitope to which the patient has generated IgE, e.g. in the case of cooked versus raw foods. Other factors may also affect the results of the test. In the case of skin tests, certain medications taken before the test (e.g. antihistamines) may cause a false negative result.

There are a number of new tests such as basophil histamine release test being developed. In this test, bloodborne basophils are exposed to allergen *in vitro* and the resultant histamine measured. Wherever the allergen cross-links basophil-bound specific IgE, the basophils will be activated and release histamine. Again, in time, such testing will be used to support other clinical information in diagnosis of IgE-mediated food allergy. The gold standard for diagnosing food allergy is the eliciting of symptoms using an oral food challenge

The oral food challenge

The oral food challenge allows a clear diagnosis of food allergy if positive. The implementation and interpretation needs to be carried out with great care and usually in conjunction with other diagnostic tests as well as the clinical history. The challenge itself should ideally be double-blind and placebo-controlled (i.e. neither patient nor clinician knows if the active or placebo food is being administered). The test needs to be conducted in a controlled, 'safe' environment, following a period in which the patient has not consumed the food being tested. Particularly in the case of IgE-mediated food allergy, a severe and even life-threatening response may be elicited. For this reason, it may be that the clinician will decide to use a clear clinical history and the presence of specific IgE as sufficient grounds for diagnosis. Care must be taken with the design of the food allergen; it should be undetectable by appearance, taste or smell and should be in the same form and type as it was for eliciting symptoms, e.g. raw versus cooked, and it must be tolerably palatable. The test should be continued until a positive response is elicited or until a 'safe' amount has been tolerated.

It can be difficult to identify a positive reaction to a food challenge. The clinician is looking for reproducible or persistent objective symptoms, however, symptoms such as flushing, vomiting or wheezing can be subjective and many symptoms may be transient or intermittent. A false positive oral challenge may be obtained if another causative food is consumed during the test or follow-up period, or if augmenting factors are present during the test, e.g. alcohol, hormonal status (menstruation), respiratory or gastrointestinal infections, psychological factors (stress). A false negative result may be obtained if an interfering drug is taken before or during the test period (antihistamine); if short-term oral-specific tolerance is being induced as increasing amounts of the food is given in the challenge; if the follow-up period is too short (especially in non-IgE-

mediated food allergy); if a cumulative dose is required to trigger symptoms. The taking of a detailed clinical history should give an indication of dose and time to response to guide the design of the food challenge. In summary, the double-blind placebo-controlled food challenge is the gold standard for diagnosis of food allergy, however, it must be designed, conducted and the results interpreted with care.

23.3.4 Management

There is no cure for IgE-mediated food allergy. Once identified, the causative allergen must be eliminated from the diet. This may require a lifelong avoidance diet or may be for a limited duration. For many IgE-mediated-food allergic patients the condition will resolve with time. The natural history of food allergy is dictated by a number of factors including the age of onset, causative allergen and co-existing conditions. Early onset allergy is more likely to be transient, but this is influenced by the allergens. Thus for IgE-mediated cow's milk allergy approximately 79% of infants will reach resolution by the age of 16 years (Skripak *et al.*, 2008), whereas for peanut allergy only 20% will reach resolution (Burks, 2008). Therefore, under clinical advice, it is possible for patients to reintroduce the offending food if there is evidence that immunological tolerance has been established. This is sometimes indicated when an inadvertent exposure to the food has not elicited any symptoms. The reduction or absence of specific IgE as indicated by repeated measures of circulating specific IgE or skin test response also suggests that resolution has occurred. The reintroduction of the food must be carefully conducted by an experienced clinician in a safe environment as a severe adverse response may be elicited.

Allergen avoidance diets are difficult to adhere to and have a very significant negative effect on the quality of life. There has been a move to improve food labelling in order to allow patients and their carers to more readily identify the presence of the most important food allergens. The European Union has identified a list of foods as cow's milk, fruits, legumes (especially peanuts and soybeans), eggs, crustaceans, tree nuts, fish, vegetables (celery and other foods of the *Umbelliferae* family), wheat and other cereals, lupin, molluscs (Commission Directives 2003/89/(EC 2003) and 2006/142/((EC)). Based on the directive, the UK Food Standard Agency lists 14 food allergens which have to be indicated by reference to the source allergen whenever they, or ingredients made from them, are used at any level in prepacked foods, including alcoholic drinks. The list consists of cereals containing gluten, crustaceans, molluscs, eggs, fish, peanuts, nuts, soybeans, milk, celery, mustard, sesame, lupin and sulphur dioxide at levels above 10 mg/kg, or 10 mg/litre, expressed as SO₂.

The successful implementation of avoidance diets requires specialised dietetic advice as well as support and encouragement. Failure to avoid the allergen risks the occurrence of a severe or even fatal reaction for the patient. In addition, the avoidance of foodstuffs which constitute the main or only dietary source of an important nutrient without proper dietetic advice (e.g. dairy products and calcium)

can result in poor health outcomes (e.g. growth and development in infants; reduced bone mineralisation and tooth decay in adults).

In tandem with allergen avoidance the administration of other medication may be necessary to manage IgE-mediated food responses. Antihistamine medications (i.e., diphenhydramine or cetirizine) are used to block the effects of histamine in IgE-mediated food allergy. These may be given orally or intravenously, if necessary, in a clinical setting. Emollient and steroid creams may be used to improve skin hydration and integrity and reduce inflammation for patients with atopic dermatitis. Respiratory symptoms of cough and wheeze can be treated by administration of a bronchodilator (e.g. the β_2 -agonist, albuterol). This may be given from a metered-dose inhaler (the efficacy of which is improved by a 'spacer' device) or by nebuliser. A course of oral steroids (e.g. the corticosteroid, prednisone) may be necessary for a short duration (two to three days) following the reaction. In the treatment of anaphylaxis, rapid administration of adrenaline (epinephrine) is necessary. This is given as an intramuscular injection and may be followed by intravenous infusion of adrenaline in the clinical setting.

An autoinjector of adrenaline is prescribed for all food allergic patients deemed at risk of anaphylaxis. The at risk patients include those with a history of previous anaphylaxis, patients with food allergy and asthma, and patients with food allergy to allergens known to be associated with more fatal and near-fatal allergic reactions (peanut, tree nuts, fish and crustacean shellfish). The adrenaline must be carried with the patient at all times to allow rapid administration by the patient or carer at the earliest signs of anaphylaxis. In addition, following the administration of adrenaline, an anaphylactic response may also be treated with antihistamine medications, β_2 -agonists, and corticosteroids. Other support therapy may be offered in the hospital such as intravenous fluids, oxygen and medications to optimise heart rate and blood pressure.

23.3.5 Future strategies

Immunotherapy is the exposure of an allergic patient to a small and increasing dose of the causative allergen in order to induce tolerance or desensitisation to the allergen. The outcome may be desensitisation, no change in allergic response, or even an increase the response. It carries the risk of precipitating a severe reaction to the therapy so must be conducted with enormous care. Subcutaneous immunotherapy has been used with some success in pollen and insect venom allergy but has been shown to be unsafe in food allergy (e.g. for peanut) (Nelson *et al.*, 1997). Sublingual or oral immunotherapy has been used experimentally for food allergic patients. There seems to be less chance of a severe response with immunotherapy administered orally. Early results suggest some success with evidence of desensitisation in studies of food allergy, e.g. peanut, milk and egg (Buchanan *et al.*, 2007; Staden *et al.*, 2007; Hofmann *et al.*, 2009; Blumchen *et al.*, 2010). However, concerns remain regarding the safety of oral immunotherapy. Furthermore, it is unclear how long this desensitising effect can be maintained and whether long-

term immune tolerance in the absence of a regular daily immunotherapy dose can be induced.

There is ongoing research investigating ways to modify the allergen used for immunotherapy to make it more likely to induce tolerance and less likely to induce IgE binding and a severe allergic reaction. One such strategy is the synthesis of short, overlapping allergen peptide sequences which span the entire allergen sequence but are in themselves too short to cause cross-linking of specific IgE. Another strategy, again to reduce the risk of IgE binding, has been to identify and mutate the IgE-binding sites of the allergen whilst preserving its ability to stimulate T cells. Results from an animal model of peanut allergy have shown results suggestive of decreased Th2 response and increased tolerance using such mutant allergens (Li *et al.*, 2003). Other researchers have used immune stimulatory sequences known to elicit Th1 responses in a murine model of peanut allergy. Early results suggest that repeated treatment with this immunostimulatory sequence prior to peanut allergen challenge in sensitised mice protected against peanut-induced anaphylaxis and reduced plasma peanut-specific IgE when compared with control animals (Kattan *et al.*, 2003). However, whether this would work in patients with established food allergy is not clear.

The neutralising of IgE is another strategy which is still being developed. Anti-human IgE antibody therapy has shown some promise with the therapy allowing a significant increase in the tolerated dose of peanuts for patients with peanut allergy (Leung *et al.*, 2003; Sampson *et al.*, 2011). Thus, although it may not prevent food allergy, it may offer some protection against a severe reaction to the accidental ingestion of a small quantity of allergen. The use of cytokines (e.g. IL-10, transforming growth factor (TGF)- β) administered to reduce food allergy has been investigated in animal models. In a murine model of egg allergy, the oral administration of TGF- β reduced IgE titres and immediate-type skin reactions (Ando *et al.*, 2007).

Traditional Chinese herbal remedies have been used to treat people for thousand of years and are still used to treat allergic patients today. Recently a nine-herb preparation (FAHF-2) has been used for testing in animal models of peanut allergy. In one study, the animals treated with the Chinese herbal preparation were completely protected against anaphylactic response to peanut allergen challenge (Qu *et al.*, 2007). The animals treated with the Chinese herbal preparation had significantly lower levels of peanut-specific IgE and lower *in vitro* Th2 cytokine response. Furthermore, animals had an increased IFN- γ production (a Th1-type cytokine which inhibits the differentiation of naïve T cells to Th2 cells) (Qu *et al.*, 2007). The blocking of this IFN- γ significantly attenuated the protective affect of the Chinese herbal preparation against peanut-induced anaphylaxis. This preparation is now being used in clinical trials in the USA.

23.3.6 Prevention

Much investigation has been carried out into prevention of allergy. Given that allergic sensitisation and symptoms can often present very early in life, this

research has been focused at the antenatal and early life period. Dietary exposure to the allergen has been studied in the maternal diet during pregnancy and lactation and in the infant diet during breastfeeding and at weaning. Also, the effects of breastfeeding versus formula feeding and breastfeeding duration and timing of weaning on development of allergy have been studied. Low-allergen formulae have been given to babies at risk of developing allergy. Another route of investigation has been to identify other lifestyle factors which may affect the propensity of the baby to develop allergy.

Maternal dietary allergen avoidance during pregnancy and lactation has been suggested as an allergy prevention method. However, there is no clear evidence that maternal avoidance of food allergens (milk, egg, fish) during pregnancy and/or lactation reduces the incidence of allergy in the offspring. Considering the possible detrimental effects on mother and baby of a nutritionally-deficient diet caused by such avoidance, the current consensus amongst experts is that mothers should not restrict their diet during pregnancy and lactation to reduce allergy in their offspring (Boyce *et al.*, 2010).

There have been some changes in advice regarding avoidance of peanut during pregnancy and lactation. As peanut allergy is often particularly severe and more likely to persist for life, advice was given for peanuts alone. In the UK, based on findings from the Committee on Toxicity, the Food Standards Agency recommended that pregnant mothers expecting a baby with a higher risk of developing allergy (e.g. those with a first degree relative with allergy) should avoid eating peanuts during pregnancy and lactation. However, this advice has been revoked after reviewing current evidence and mothers are no longer advised to avoid peanuts during pregnancy and lactation even if they have allergic disease within the family (Hourihane *et al.*, 2007). However, a recent study was conducted of 503 babies in the USA with likely milk or egg allergy but no evidence of peanut allergy. In this cohort of infants, frequent maternal peanut consumption during pregnancy and lactation was dose-related to infant peanut-specific IgE (Sicherer *et al.*, 2010). Furthermore, in a group of 71 infants who were never breastfed, maternal peanut consumption during pregnancy was strongly associated with high infant peanut-specific IgE (5 kU/L) (Sicherer *et al.*, 2010). It may be that this early sensitisation is not followed by the early period of oral tolerance with peanut. In a study of peanut allergy in the UK and Israel, it was found that early peanut consumption (8–14 months of age) was far greater in the children in Israel (mean 7.1 g/month) than in the UK (mean 0 g/month), but that peanut allergy was 10-fold greater in the UK infants (Du Toit *et al.*, 2008). A UK-based study of over 600 infants investigating peanut allergy (Learn Early About Peanut allergy or LEAP study) is currently underway and may help to address some of these controversies.

Evidence that breastfeeding protects against the development of allergy in the infant is inconclusive. There are, of course, many other good reasons to advocate breastfeeding for the health of the baby. There have been numerous studies looking at the allergic outcome of breastfed versus formula fed infants. Most have found no significant effect with a few finding positive and even negative effects of breastfeeding on the allergic status of the infant.

There is some evidence that the use of low-allergy formulae can prevent the onset of allergic disease to cow's milk in at risk infants. These formulae may be soy milk rather than cow's milk based, or the milk may be treated to reduce its allergenicity (hydrolysed), or based on amino acids rather than protein. These formulae are used with success to treat cow's milk allergic infants but are very expensive. Thus it is not clear whether it is cost effective to use this as a prevention strategy and whether the benefit will be long-term.

Few studies exist looking at the effects of timing of solid food introduction and the development of food allergy. There have been some results to suggest that early introduction of solids (<4 months) may increase the incidence of food allergy. However, there is no evidence for supporting delayed introduction of solids, and late introduction of solid foods may cause problems with feeding and optimal nutrition in the developing infant.

The rising prevalence of food allergy has prompted investigation into changes in lifestyles over the last few decades. The 'hygiene hypothesis' is based on the idea that we are no longer exposed to the array of micro-organisms in early life which we would have been a few decades ago. Although advantageous in some ways, it is suggested that this may affect the early programming of the immune system. The presence of commensal bacteria in the gut provides special signals (microbe-associated molecular patterns) to the innate immune system via pathogen recognition receptors on the intestinal epithelial cells (Jarchum *et al.*, 2011). This signalling allows the innate cells (e.g. APCs such as macrophages and dendritic cells) to give the 'correct' signals to the adaptive immune cells (including T and B cells) when encountering antigens, thus avoiding the generation of a food allergen-specific Th2 response. Thus the establishment of a normal healthy gut microbiota is thought to be critical in influencing the immune system to maintain tolerance to food antigens and balance the Th1 versus Th2 immune response (McLoughlin and Mills, 2011). This has generated much research into early lifestyle and dietary factors which may influence the early establishment of the gut microbiota and thus the susceptibility of infants to develop allergen-specific IgE and allergic symptoms.

A change in the dietary intake of types and amounts of fats has also been postulated as a reason for the increased prevalence of allergy. The increased intake of omega 6 polyunsaturated fats from some vegetable oils and margarines (e.g. from corn, safflower and sunflower oil) in the last few decades has been suggested as a possible cause for the increased incidence of allergy (Black and Sharp, 1997). Omega 6 fatty acids such as arachidonic acid are incorporated into the membranes of immune cells and can be released from the membranes to act as substrate for a number of immune mediators (leukotrienes, prostaglandins). These mediators elicit a number of effects on the immune system including inducing IgE production and allergic inflammation. The closely related omega 3 family fatty acids (e.g. eicosapentaenoic acid, found in oily fish and fish oil) can reduce mediator production from the omega 6 fatty acids. There are a several studies showing that increased fish oil or oily fish intake in pregnancy reduces the levels of sensitisation to food allergens and may reduce early atopic dermatitis (reviewed in Kremmyda

et al., 2011). However, more studies and longer term follow-up is needed to substantiate the data.

The role of vitamin D in early life and the development of allergy is another area of interest. Dietary sources of vitamin D are limited, but exposure to sunshine allows synthesis of vitamin D in the skin. Lifestyle changes in the last few decades (less time spent outside and use of sunblock cream and protective clothing to prevent sunburn and related cancers) have meant that sunshine exposure is significantly reduced. Camargo *et al.* (2007a) noted that prescriptions of adrenaline autoinjectors (used to treat anaphylactic reactions) in the USA in 2004 were significantly more common per head of population in the northern than more southern states and interpreted this as an inverse relationship between sunshine (as a proxy for vitamin D status) and anaphylaxis (e.g. severe IgE-mediated allergy). Several studies have looked at maternal vitamin D intake in pregnancy and allergic outcome in the offspring (Camargo *et al.*, 2007; Devereux *et al.*, 2007; Erkkola *et al.*, 2009). Although there appear to be some protective effects of an increased maternal intake of vitamin D during pregnancy and infant respiratory symptoms there is currently no information on the relationship with food allergy in the offspring.

In summary, there are currently no methods for preventing the development of IgE-mediated food allergy although some areas of research have shown early promise.

23.4 Non-IgE-mediated and mixed- or IgE- and non-IgE-mediated food allergy

There are a number of different allergic conditions caused by immune responses which are not primarily IgE-mediated. These include responses in which allergen-specific-IgE is normally absent (i.e., food protein-induced allergic proctocolitis and food protein-induced enterocolitis syndrome) and also conditions, sometimes termed mixed mediated food allergy (e.g. eosinophilic oesophagitis and eosinophilic gastritis), in which allergen-specific IgE is often present but is not the major mediator of the pathology in the same way as classical IgE-mediated immediate hypersensitivity reactions (Fig. 23.2). Most of these conditions present as symptoms of the gastrointestinal tract along with features of malabsorption. However, amongst the non-IgE-mediated allergic responses to cow's milk is a rare pulmonary condition, Heiner syndrome. This presents as chronic or recurrent lower respiratory symptoms and in severe cases, pulmonary haemosiderosis (intra-alveolar haemorrhage leading to abnormal iron accumulation). The elimination of milk from the diet results in an improvement in symptoms within days and clearing of pulmonary infiltrated within weeks (James, 2003).

23.4.1 Immune mechanisms

The mechanisms causing mixed and non-IgE-mediated food allergies are not well understood. In eosinophilic oesophagitis and eosinophilic gastritis where IgE is

also involved, there is a marked infiltration of the oesophagus, stomach and/or intestinal walls with eosinophils, basal zone hyperplasia and papillary elongation. In about half of the cases of eosinophilic oesophagitis and eosinophilic gastritis, peripheral eosinophilia is present. The eosinophilic infiltration may involve the mucosal, muscular or serosal layers, and the extent of the infiltration may correlate positively with the severity of symptoms. Mast cells also appear to have a role in eosinophilic oesophagitis. The expression of mast cell-related genes such as those for the high-affinity IgE receptor, the histamine-synthesising enzyme, histidine decarboxylase and two mast cell-specific proteases were upregulated in oesophageal biopsies from adult eosinophilic oesophagitis patients compared with age-matched controls. Symptom resolution in these patients by successful food elimination or the use of steroids significantly reduced the expression of these mast cell-specific genes (Hsu Blatman *et al.*, 2011). Peripheral blood T cells from patients with these eosinophilic conditions secrete significantly more of the Th2 cytokines, IL-4 and IL-5, than T cells from controls. Recent studies have also demonstrated that eotaxin-3, a potent eosinophil attractant (Bhattacharya *et al.*, 2007), and IL-13 may have important roles in the pathology of eosinophilic oesophagitis (Blanchard *et al.*, 2007).

There is very limited information on the underlying causes of other non-IgE-mediated food allergy. In infants with food protein-induced enterocolitis syndrome, peripheral blood mononuclear cells cultured with food antigens have been demonstrated to have a higher proliferative response when compared to those from controls (Powell *et al.*, 1989). Furthermore, peripheral blood mononuclear cells cultured with food antigens from food protein-induced enterocolitis syndrome patients produce larger quantities of tumour necrosis factor (TNF)- α over a more prolonged period than those from age-matched control children (Benlounes *et al.*, 1999). Furthermore, increased expression of TNF- α has also been detected in the duodenal biopsies of children with food protein-induced enterocolitis syndrome, along with a decreased expression of the receptor for TGF- β 1 (Chung *et al.*, 2002). This suggests that increased TNF- α , along with reduced tolerance which may be partially mediated through reduced TGF- β signalling, may be involved in the immune response of this non-IgE-mediated food allergy.

There is a paucity of information regarding the immune mechanisms causing food protein-induced enteropathy. An increase in the numbers of CD4⁺ T cells in the small intestine along with the presence of intraepithelial CD8⁺ cells which normalise during elimination diets (Savilahti, 2000) is suggestive of some of the mechanisms seen in CD.

23.4.2 Presentation

Eosinophilic oesophagitis and eosinophilic gastritis

Eosinophilic oesophagitis and eosinophilic gastritis is often seen in infancy and childhood but may present at any age. It typically presents as a persistent, chronic

reflux or vomiting disorder with some of the following features: poor appetite; abdominal discomfort or pain; food refusal; sleeplessness; irritability; diarrhoea; failure to thrive. In older children postprandial vomiting, anorexia, haemtaemesis, dysphagia (not able to eat), weight loss and poor growth and development may be apparent. As the condition progresses, food impaction may occur due to oesophageal strictures and oesophageal dysmotility. Frequently polysensitisation to a number of different food types is present. Common trigger foods include milk, soy, eggs and wheat. The condition is likely to be persistent.

Food protein-induced enterocolitis syndrome

Food protein-induced enterocolitis syndrome typically presents in the first several months of life. Infants may be irritable, lethargic, have protracted bouts of vomiting and diarrhoea, and may be dehydrated. Vomiting usually occurs within one to four hours of feeding. If untreated, symptoms may include bloody diarrhoea, anaemia, abdominal distention and failure to thrive. In around 15–20% of infants, pronounced dehydration and hypovolaemic shock is seen. Food protein-induced enterocolitis syndrome has not been reported in exclusively breastfed infants, with reactions to foods being reported at weaning, i.e. triggering allergen ingestion is not via the breastmilk (Nowak-Węgrzyn and Muraro, 2009). Causative foods are frequently cow's milk or soy protein-based formulae. Around half of infants who are symptomatic in response to cow's milk will also react to soy (Nowak-Węgrzyn and Muraro, 2009). Infrequently, food protein-induced enterocolitis syndrome may be caused by solid foods such as rice, egg, wheat, oat, peanut, chicken, turkey, fish and other foods, especially when presenting in older children. In adults similar symptoms may be seen, often in response to crustacean shellfish (shrimp, crab and lobster). Resolution of food protein-induced enterocolitis syndrome is variable depending on factors such as the causative allergens and the age of diagnosis. The majority of cow's milk-sensitive patients (60%) will lose sensitivity by three years of age (Sicherer *et al.*, 1998; Nowak-Węgrzyn *et al.*, 2003). Data for resolution for other allergens by the age of three years include 40% for rice, 66% for oats and 67% for vegetables (Nowak-Węgrzyn *et al.*, 2003).

Food protein-induced (allergic) proctocolitis

This is an allergic inflammatory process involving the distal colon. It presents typically in the first few months of life as low-grade rectal bleeding, often in an apparently well infant. A typical feature is the presence of stools streaked with blood and mucus. Occasionally, the infant may be anaemic. Interestingly, over half of the infants presenting with food protein-induced proctitis are breastfed, sometimes exclusively. Symptoms are usually induced by cow's milk proteins contained in formula or breastmilk, but may be caused by egg or other food proteins (soy, rice, wheat). Symptoms usually resolve within a few months. In cow's milk protein-induced proctocolitis milk can usually be successfully reintroduced between six and 12 months of age.

Food protein-induced enteropathy

Food protein-induced enteropathy is typically more insidious in presentation. The onset of symptoms may be seen 40–72 hours after ingestion of the allergen. Symptoms include persistent diarrhoea (often steatorrhea), perianal excoriation, vomiting, irritability and failure to thrive or weight loss. Oedema and ascites may be in evidence in severe cases due to enteric protein loss. There may be indications of malabsorption such as deficiencies in iron or vitamin K. In common with other food-sensitive enteropathies such as CD, the damage to the enterocytes may cause secondary lactose intolerance, due to lactase insufficiency, and associated lactose-induced diarrhoea. The causative food is often cow's milk but may be due to other foods such as wheat, eggs or soy. Food protein-induced enteropathy is often transient and most infants with this condition will become symptomatic by 12 months of age and will reach resolution by two to three years of age.

23.4.3 Diagnosis*Eosinophilic oesophagitis and eosinophilic gastritis*

In patients with eosinophilic oesophagitis there is frequently concomitant atopic disease, often with respiratory symptoms, and many have a family history of allergy. Patients with seasonal rhinitis (hay fever) have seasonal variation in both oesophageal eosinophils and related symptoms. Food induced anaphylaxis occurs in around 15% of eosinophilic oesophagitis patients (more likely in pediatric cases), again linking eosinophilic oesophagitis with IgE-mediated hypersensitivity. Endoscopic biopsies show high eosinophilia and mild mastocytosis. However, uneven distribution of eosinophil infiltration and deeper infiltration (of muscular and serosal layers) may mean that the biopsies do not permit detection of eosinophilia. To facilitate diagnosis, the examination of at least three full-thickness biopsies is recommended (Furuta *et al.*, 2007). Some degree of eosinophilia (between one and six eosinophils per high-powered field) may also be seen in the oesophagus of patients with gastroesophageal reflux disease. Thus, to clarify the diagnosis, a criterion of 15 eosinophils per high-powered field has been suggested as diagnostic for eosinophilic oesophagitis. However, in cases where the number of eosinophils may be above six but below 15 per high-powered field some confusion may exist. Allergen-specific IgE may be detected by blood test or skin prick test, but this is not always helpful as causative foods may not be detected, and specific IgE may be detected to foods which do not cause symptoms.

Atopy patch testing has also been used in some studies. In this test food allergen is placed on intact skin, covered and left for 48–72 hours. This test looks for a cell-mediated response to the allergen. However, the test is not well standardised and does not reliably identify the causative allergen. Thus it may or may not be of help in management of the problem. The successful elimination of the causative foods from the diet, followed by an oral food challenge, is the best means of diagnosis, if safe and practicable. Histological examination of repeat biopsies taken during

elimination and after reintroduction of the offending food can give clear evidence of the diagnosis.

Food protein-induced enterocolitis syndrome

This syndrome can be hard to recognise. Nowak-Wegryzn and colleagues (2003) suggested the following criteria for diagnosis:

- (a) A repetitive vomiting response and/or diarrhoea within 4 hours of ingestion (following first exposure to a food) in the absence of other identifiable causes.
- (b) Symptoms limited to gastrointestinal tract.
- (c) Complete resolution of symptoms on avoidance.
- (d) Recurrence of symptoms upon challenge.

If taken, biopsies of the jejunum show flattened villi, the presence of oedema and infiltration of lymphocytes, eosinophils and mast cells. Stool samples may show the presence of large numbers of leukocytes and eosinophils. Most patients with food protein-induced enterocolitis syndrome will not demonstrate allergen-specific IgE by skin prick test or blood test. Again the gold standard for diagnosis of food protein-induced enterocolitis syndrome is the reproduction of clear symptoms during an oral food challenge. However, given the severity of the response in some patients (particularly hypovolaemic shock), this may be considered neither safe nor desirable. Thus the diagnosis will often rely upon the clinical history and significant symptom improvement when maintaining strict allergen avoidance.

Food protein-induced (allergic) proctocolitis

Diagnosis is made from the clinical history and presentation. Allergen-specific IgE is normally absent and thus not reliably useful for diagnosis, although there may be a history of atopy. Rectal biopsies show eosinophilic infiltration and inflammation and, in some cases, erosions with microabscesses or fibrosis. However, rectosigmoidoscopy and biopsy are not recommended for diagnosis on a routine basis. Usually, the presentation, case history and response to elimination of the allergen from the diet form the diagnosis, following ruling out of other possible causes for the symptoms (e.g. infections, necrotising enterocolitis, anal fissures). An oral food challenge may be conducted.

Food protein-induced enteropathy

The case presentation and response to dietary elimination of the causative allergen are the mainstays of diagnosis. Allergen-specific IgE is normally absent and thus not reliably useful for diagnosis and management. Endoscopic biopsies may be taken for examination. If the histology indicates that enteropathy is present there is need to differentiate the cause from other likely reasons such as infectious enteropathy (especially Giardiasis), CD and lactose intolerance. There may be confusion with the diagnosis of lactose intolerance as secondary lactose intolerance may be caused by the pathology in the small intestine. This confusion can be augmented by the partial improvement of symptoms on a lactose-free diet as a result of a reduction in lactose-induced osmotic diarrhoea.

23.4.4 Management

Eosinophilic oesophagitis and eosinophilic gastritis

Elimination diets are the mainstay of treatment for eosinophilic oesophagitis or gastritis, removing foods causing symptoms from the diet. However, such diets can be difficult to implement because of multiple sensitisations and difficulty in identifying causative foods, which may include both common and uncommon food types. Elemental diets (amino acid-based) can be very effective, but they are not well tolerated and very expensive. They are particularly hard to maintain in older children or adults. For infants an amino acid-based formula is used.

Anti-acid therapy (proton-pump inhibitors) is given initially. This is given to reduce the gastric hyperacidity which aggravates, rather than causes, the eosinophilic inflammation seen in eosinophilic oesophagitis. Systemic (acute exacerbations) and topical (long-term control) glucocorticoid therapy are successful in the eosinophilic oesophagitis and gastritis patients. In a small proportion of patients, steroid therapy is ineffective, probably due to corticosteroid resistance. Eosinophilic oesophagitis is normally chronic; thus therapy needs to be long-term and cessation is normally associated with the return of symptoms.

In eosinophilic oesophagitis, oesophageal strictures may be present and, if causing severe problems for the patient's ability to swallow, may require physical dilatation. This procedure must be carried out with great care as there is a significant risk of perforation. The administration of topical steroids prior to the procedure may reduce the inflammation and thus the risk of perforation.

Food protein-induced enterocolitis syndrome

The mainstay of treatment for food protein-induced enterocolitis syndrome is elimination of all sources of the food allergen from the diet. For infants an extensively hydrolysed cow's milk formula or an amino acid-based formula may be used. Symptoms abate for most patients within three to ten days. If the infant presents with hypovolaemic shock, intravenous fluids must be administered to rehydrate and corticosteroids may also be given to help suppress the cellular immune response. Reintroduction of the causative food allergen may be attempted at regular intervals (every 18–24 months). This needs to be carried out in a safe clinical setting and with great care, being particularly mindful of the risk of inducing hypovolaemic shock.

Food protein-induced (allergic) proctocolitis

The majority of patients with food protein-induced allergic proctocolitis respond rapidly to elimination of the food allergen from the diet or, in the case of breastfed infants, from the maternal diet. Usually resolution of symptoms will be apparent within 48–72 hours. If this is not the case, the use of an elemental amino acid-based formula diet can be used. This should be a short-term intervention as most infants are able to tolerate the causative allergen by nine months of age (Lake, 2000).

Food protein-induced enteropathy

In common with the other food allergies, food protein-induced enteropathy must

be treated with the implementation and maintenance of a strict allergen avoidance diet. As resolution is achieved in the majority of infants presenting with this condition by 3 years of age, careful re-introduction of the offending food may be attempted when deemed appropriate.

23.4.5 Future strategies

Novel strategies which could be used for the treatment or management non-IgE-mediated or mixed response-mediated food allergy include those directed towards induction of oral tolerance to the specific food using immunotherapy such as those already being trialled in IgE-mediated food allergy (see Section 23.3.5) (Burks *et al.*, 2008). The use of cytokine modulation to induce tolerance may also offer a novel therapeutic approach. In a murine model of food allergy, the introduction of bacteria engineered to produce murine IL-10 increased murine IL-10 and IgA production (Frossard *et al.*, 2007). Although the model is based on IgE-mediated response, such immune manipulation may ameliorate or protect against the breakdown of immune tolerance seen in non-IgE or mixed response type food allergic reactions.

Montelukast has been used to reduce eosinophil inflammation in patients with eosinophilic oesophagitis with some success (Attwood *et al.*, 2003). This is a cysteinyl leukotriene receptor antagonist which blocks the action of leukotriene D₄ on eosinophils. Patients with eosinophilic oesophagitis have been treated with an anti-IL-5 antibody, given as three infusions on a monthly basis in an experimental trial (Stein *et al.*, 2006, 2008; Straumann *et al.*, 2008). This therapy resulted in reduced symptoms, improved quality of life and a significant fall in eosinophil counts (Garrett *et al.*, 2004). There are further studies investigating this therapy in children.

23.4.6 Summary

Mixed and non-IgE-mediated food allergy describes a number of food allergic responses in which type I, immediate IgE-mediated hypersensitivity is not the causative mechanism. These allergies are often difficult to diagnose with no clear diagnostic tests available. Perhaps because of this, mixed and non-IgE-mediated food allergies have received investigative attention for a shorter time period than IgE-mediated food allergy and are less well understood. In common with IgE-mediated food allergy there is no current cure for these responses. Allergen avoidance remains the only really effective management strategy.

23.5 Coeliac disease

23.5.1 Introduction

Coeliac disease (CD) is also known as non-tropical sprue or gluten-sensitive enteropathy. It is an autoimmune reaction causing severe damage to lining of the

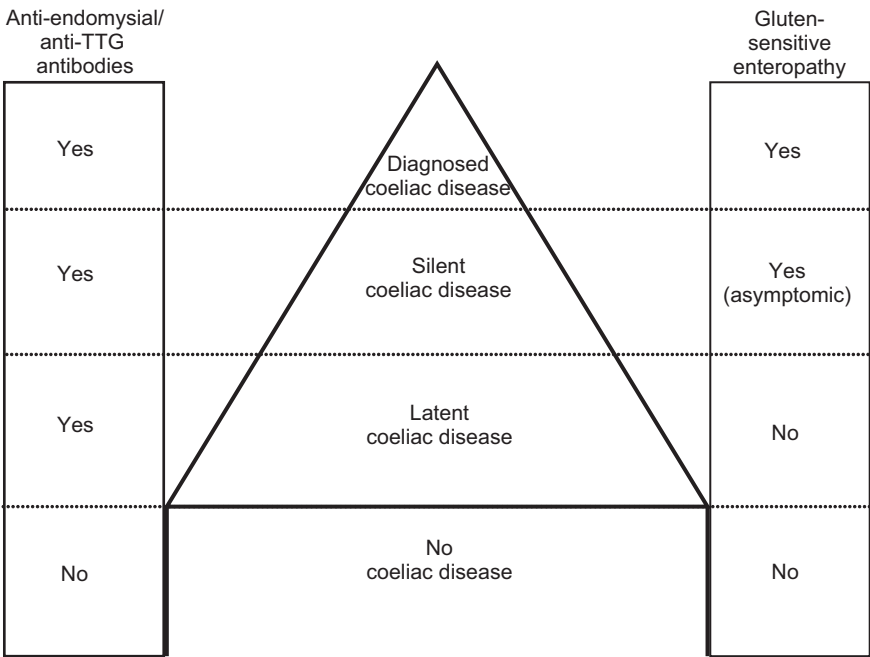


Fig. 23.4 The majority of coeliac disease (CD) is present in a subclinical form. There are people who have CD-associated antibodies but who have no symptoms or small intestine pathology (latent), those who have CD-associated antibodies, have no symptoms but have some degree of small intestine pathology (silent) and those who have CD-associated antibodies and are symptomatic with the characteristic small intestine pathology. TTG, tissue transglutaminase.

small intestine which is precipitated by the development of an immune response to cereal antigens such as those found in gluten from wheat. It was first described in 1888 by an English physician, Samuel Gee, who recognised the syndrome but did not make the connection with the food antigens which drove the response. It took the astute observation of a paediatrician, Willem-Karel Dicke, and the severe Dutch famine during World War II to make this connection. He noticed that during the worst periods of the famine, children with CD had a reduced mortality rate. After the war, when refeeding programmes addressed the national malnutrition the mortality rate amongst children with CD increased to the former rate (Dicke *et al.*, 1953). By a process of elimination he identified wheat-containing products as the cause of their symptoms.

Previously considered to be a very rare disease, CD is currently estimated to affect approximately 1% of the population in the UK, Europe and USA. It was the introduction of technologies allowing serological testing for antibodies in people with CD which allowed a clearer estimation of the prevalence of CD. There is still concern over the accuracy of the true prevalence of the disease, as much CD is present in a subclinical form. Thus, there are people who test seropositive for CD-associated antibodies who have no symptoms or small intestine pathology (latent),

those who are seropositive, have no symptoms but have some degree of small intestine pathology (silent), and those who are seropositive and are symptomatic with the characteristic small intestine pathology (Fig. 23.4). The relationship between the 'latent' and 'silent' seropositive CD individuals and those who have clinical CD is not clear. Some individuals with a 'silent' or 'latent' status will undoubtedly develop clinical CD at some point, but which individuals will, and what the precipitating factors might be, remains to be determined.

23.5.2 Immune mechanisms

The antigen

The first antigen which was identified as eliciting the immune response in CD was a component of gluten from wheat. Gluten is a storage protein contained within the wheat endosperm. Gluten confers the sticky, elastic texture of wheat dough, giving the characteristic structural properties when cooking or baking wheat flour-based doughs. Not only is this viscoelastic property of gluten extensively used in the bakery and pasta-making industry, but it is also used as a binder in many processed foods. Gluten represents approximately 80% of the proteins in wheat endosperm. The relative solubility in water and alcohol can be used to separate gluten into two fractions: the polymeric glutenins and the alcohol-soluble, monomeric gliadins (Fig. 23.5). Gliadins are the major wheat-derived antigens driving the immunogenic response in CD sufferers. In some cases of CD, immune reactivity to glutenin components can also be seen. Similar storage proteins (prolamins) exist in other food grains such as rye (secalin), barley (hordein) and oats (avenin). Thus foodstuffs containing wheat, barley or rye will cause CD symptoms. There are also reports of sensitivity to oats in some CD patients.

Antigen handling

The development of an antigliadin immune response is partially dictated by genetics. Evidence for this includes the high concordance of CD between monozygotic twins (80%) and the fact that having a family member with CD significantly increases an individual's risk of having CD (Dube *et al.*, 2005; Nistico *et al.*, 2006). The strongest genetic link is conferred by the type of antigen presenting molecules carried. These molecules are the means by which the immune system 'introduces' or 'shows' an antigen to other cells of the immune system. CD is far more likely to present in individuals who carry particular antigen presenting molecule types. So almost all (97%) CD patients will carry either human leukocyte antigen (HLA) DQ2 or DQ8. Also there is a dose effect whereby people who are homozygous for HLA DQ2 are more susceptible to developing CD compared to heterozygous carriers of HLA DQ2 (Mearin *et al.*, 1983). The severity of CD and associated clinical conditions was demonstrated to be greater in people who are homozygous rather than heterozygous for HLA DQ2 (Al-Toma *et al.*, 2006). However, not all individuals carrying HLA DQ2 or DQ8 will have CD, demonstrating that these HLA genotypes are necessary but not sufficient to allow the development of CD.

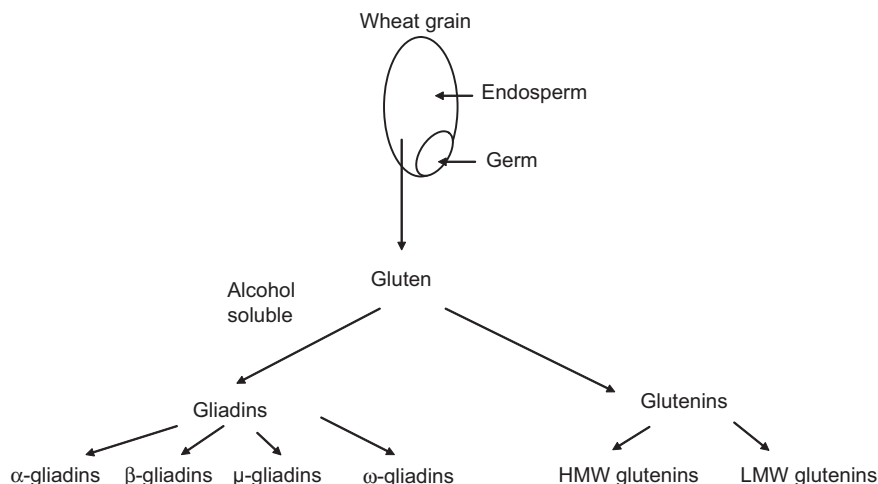


Fig. 23.5 Storage proteins (gluten) constitute approximately 80% of the proteins in wheat. The gluten can be subdivided into the alcohol-soluble monomeric gliadins and the polymeric glutenins. The gluten fractions can be further divided by molecular weight into α -, β -, μ - and ω -gliadins and high molecular weight (HMW) and low molecular weight (LMW) glutenins. Although the gliadins, and particularly the α -gliadins, are considered the most important causative antigens in CD, other gliadin subunits and glutenins may also be toxic.

Genetics are only one of a number of factors affecting the manifestation of CD. Several other genes have been linked with CD although not as strongly as the HLA types. Most of these genes code for cytokines or cytokine receptors.

In CD the immune response to gliadin results in the generation of anti-gliadin antibodies but also antibodies against the enzyme involved in metabolism of gliadin, tissue transglutaminase (TTG). This is now an immune response to a 'self' component and is thus an autoimmune response. The reason for this autoimmune reaction is not fully understood. TTG itself is ubiquitous and is seen both intracellularly and extracellularly. It is normally quiescent and requires signals and the presence of millimolar concentrations of calcium ions before it becomes active. The signals required to activate TTG may occur during inflammation, such as that seen in infection. It has been suggested that certain infections (rotavirus) may precipitate CD symptoms in susceptible individuals (Stene *et al.*, 2006). In addition, such gastrointestinal infections would be likely to disrupt the epithelial integrity thus facilitating the uptake of gluten peptides.

Gliadin is rich in the amino acids proline (15%) and glutamine (30%). A high proportion of proline and glutamine is also found in secalin and hordein. The high proline content renders the gliadin protein resistant to breakdown by gastrointestinal protease enzymes. Therefore it remains as longer polypeptide fragments (10–50 amino acid residues) which are more immunogenic. During the metabolism of gliadin, it is deamidated by TTG, a reaction in which glutamine residues are converted into negatively charged glutamates. These negatively charged residues

increase the strength of the binding of the antigen to the antigen-carrying grooves in the HLA DQ2 and DQ8 molecules, thus increasing the opportunity for antigen presentation and thereby the antigenicity of the molecules. Therefore, both whilst in a complex with the enzyme and as the deamidated product, the gliadin is highly antigenic, driving a strong immune response.

The transepithelial uptake of gliadin peptides is increased in CD patients with active disease and is reduced by strict gluten avoidance. The gluten protein may enter the lamina propria by a number of mechanisms. There are several possible transcellular routes for uptake of gluten. Intact gluten peptides, bound to soluble IgA, can be taken up transcellularly via the transferrin receptor (CD71). Furthermore, an IFN- γ -dependent transcytosis mechanism has been shown to result in uptake of the immunodominant α -2gliadin-33mer in the lamina propria. IFN- γ is profoundly upregulated in duodenal biopsies of patients with active CD when compared with healthy patients (Nilsen *et al.*, 1998). The paracellular route via defective tight junctions may be the result of overexpression of zonulin, a protein which can disrupt tight junctions (Lammers *et al.*, 2008). This protein is now the focus of intense research for future anti-CD therapies.

Immune response and pathology

Once in the lamina propria the gluten, either in its native or deamidated form, will be taken up by APCs such as dendritic cells, and presented to cells of the immune system. This interaction generates gluten-specific CD4⁺ T cells which initiate the inflammatory response in the small intestine and provide immune help to specific B cells. Initially these B cells are induced to secrete antigluten antibodies. Ultimately the antibodies generated are directed at the gluten peptides, TTG and the endomysium.

The CD4⁺ T cells are mainly concentrated in the lamina propria but, in the epithelium of symptomatic CD patients, are increased numbers of cytotoxic intraepithelial lymphocytes (IEL). The vast majority of these IEL are CD8⁺ TCR $\alpha\beta$ ⁺ or TCR $\gamma\delta$ ⁺. Although the presence of increased numbers of gluten-specific CD4⁺ T cells will drive inflammation within the lamina propria, it is the IELs which result in the intestinal epithelium tissue damage seen in active CD. These IELs express natural killer (NK) receptors in both healthy and CD-affected small intestine. However, in active CD, there are greater numbers of IELs and they express a high level of activating NK receptors. Using an NK receptor-mediated cytotoxicity, these cells are able to lyse intestinal epithelial cells.

The NK receptor-mediated cytotoxicity is upregulated by pro-inflammatory cytokines such as IL-15 and IFN- γ and IFN- α . These cytokines are found in high concentrations in biopsies of the small intestine from patients with CD (Nilsen *et al.*, 1998; Maiuri *et al.*, 2000; Monteleone *et al.*, 2001; Di Sabatino *et al.*, 2007). Indeed, IFN- γ can be detected in concentrations 1000-fold greater in active CD than in healthy lamina propria. Normally, IL-15 production is tightly controlled and the overexpression of IL-15 increases activation, survival and cytotoxicity of IELs (Meresse *et al.*, 2004) and also inhibits the production of cytokines involved in inducing tolerance such as TGF- β (Benahmed *et al.*, 2007). The result of the

immune response is severe damage of the lining of the small intestine. This tissue damage and remodelling is characterised by loss of epithelial cells, crypt hyperplasia and villous atrophy. Interestingly, amongst the IELs are CD8⁺ TCR $\gamma\delta$ ⁺ cells which are present during active CD, but also remain in high numbers in the epithelium of patients who maintain a gluten-free diet. The presence of these cells in the mucosa, years after the active CD has resolved, suggests that they are not involved in the pathology but may be present as regulatory elements. This is supported by the finding that these cells can produce TGF- β and express inhibitory receptors (Bhagat *et al.*, 2008).

23.5.3 Presentation

CD has a biphasic time distribution of presentation with two peaks occurring, one in childhood and the other in middle age. Presentation may typically include gastrointestinal symptoms and features resultant from malabsorption. Children presenting with CD will classically demonstrate chronic diarrhoea, failure to thrive, abdominal distension or discomfort and sometimes delayed puberty. In adults with CD, typical features include chronic fatigue, anaemia, chronic diarrhoea, weight loss, peripheral neuropathy, reduced fertility, joint pain and short stature. Commonly, adults with CD will have low bone mineral density resulting from malabsorption of calcium.

Symptoms of a gastrointestinal nature (e.g. diarrhoea, abdominal discomfort or pain, vomiting) may be directly related to the effect of the enteropathy but may also be related to secondary effects of this damage. In severely affected CD patients, the loss of epithelial cells may result in a reduced lactase production and thus secondary lactose intolerance. A range of other manifestations (e.g. failure to thrive or short stature, loss of bone mass, osteoporosis, dental enamel hypoplasia, delayed puberty, chronic fatigue, anaemia) are a result of chronic malabsorption of vitamins and other essential dietary factors such as iron, folate, calcium, zinc and the fat-soluble vitamins A, D, E and K. There are also a range of associated symptoms of a neurological or psychiatric nature for which the cause is not yet identified (e.g. peripheral neuropathy, ataxia, epilepsy, especially with occipital calcifications, schizophrenia, depression, dementia, headaches and autism). Interestingly, the severity of the clinical presentation of CD does not seem to be related to the apparent severity of enteropathy, again suggesting that other mechanisms may play an important role in the manifestation of symptoms.

A number of clinical conditions are associated with CD, many of an autoimmune nature. These include type 1 diabetes mellitus, autoimmune thyroiditis, Sjogren's syndrome, systemic lupus erythematosus, autoimmune hepatitis, Addison's disease, primary biliary cirrhosis and IgA deficiency. CD is also seen at a higher frequency in people with Down's, Turner's or William's syndrome. Dermatitis herpetiformis, a rare skin rash affecting the extensor surfaces, is also strongly associated with CD and is sometimes termed CD of the skin. Dermatitis herpetiformis may present in the absence of diagnosed CD and is characterised by anti gliadin IgA in the skin and is almost always successfully treated by a strict

gluten-free diet and Dapsone. Long-term complications of untreated CD are the manifestation or continuation of manifestations of symptoms related to the enteropathy and associated malabsorption but also a reduced survival rate mostly attributable to an increased incidence of non-Hodgkin lymphoma.

23.5.4 Diagnosis

The introduction of serological testing in the 1980s revolutionised the diagnosis of CD. Up to this time, cases were diagnosed by clear intestinal-related symptoms and signs of malabsorption, confirmed by a positive duodenal biopsy. However, the presence of histopathology such as IEL infiltration or villous atrophy in a biopsy may be seen in other enteropathies and is therefore not diagnostic of CD in itself.

The severity of the enteropathy can be graded. Several schemes have been suggested, but the three grade scale devised by Corazza *et al.* (2007) offers simplicity and reproducibility amongst diagnostic staff. In this scheme there is a grading of the severity of lesions from A: Signs of lymphocyte infiltration but no villous atrophy; B1: Lesions demonstrating atrophy but still with detectable villi; B2: Lesions with atrophy and undetectable villi. The degree of pathology has important implications for the successful resolution of the condition. For most patients with symptomatic CD, morbidity and pathology will be resolved if a strict gluten-free diet is adhered to. Thus, most cases of refractory CD (unresponsive to a gluten-free lifestyle) can be addressed by the implementation of dietary investigation and advice to detect accidental (or intentional) continued consumption of gluten and advise accordingly. Additionally, some refractoriness is due to a slow recovery or misdiagnosis where common diagnostic features exist (e.g. autoimmune enteropathy). However, among patients with grade B2 lesions, there are two categories of true refractory CD. These are termed type 1 and type 2 refractory CD and are distinguished by a number of immunological and clinical features. Type 2 refractory CD is of particular concern as, in contrast to type 1, it is unresponsive to immunosuppressant therapy and is associated with a high risk of developing enteropathy-associated T-cell lymphoma and a dramatically increased five-year mortality (estimated at around 50%) (Biagi and Corazza, 2010).

The detection of anti-endomysial IgA is sensitive (around 95% of CD-affected individuals positive) and specific (around 99% of those testing positive have CD). The detection of TTG IgA is also sensitive (around 87% of CD affected individuals detected) and specific (around 95% of those testing positive have CD) (Hill *et al.*, 2005). The absence of positive serology should not preclude further testing for CD where the condition is suspected due to clinical symptoms. The frequent presentation of low serum IgA in CD-affected individuals requires that a total IgA measurement should be made along with the serological testing to avoid false negative serology resulting from a lack of IgA. If the patient presents with hypoIgA the serology can be performed looking for anti-endomysial or TTG IgG. Thus, if performed correctly, the use of serological testing for anti-endomysial antibodies or TTG antibodies confirmed by a duodenal biopsy is an accurate and cost-effective method for detecting CD. It also allows for the detection of individuals

with less severe intestinal pathology who would formerly have remained undiagnosed. It is these individuals who respond most fully to a gluten-free diet.

There is great concern amongst the medical professions that even with the current diagnostic programme, a greater awareness is needed amongst physicians to detect CD, particularly where the presentation may be atypical. The introduction of a mass screening programme, either for all individuals or targeted towards those in a higher risk category (e.g. relative with CD, or the presence of associated conditions, e.g. type 1 diabetes mellitus, autoimmune thyroiditis, Down's syndrome, Turner's syndrome, William's syndrome or IgA deficiency), has been suggested. Non-targeted screening programmes already exist for conditions such as phenylketonuria with a much lower prevalence than CD, adding weight to this suggestion.

23.5.5 Management

Gluten-free diet

Once diagnosed the management of CD requires the instigation of a lifelong gluten-free lifestyle and the screening for, and addressing of, nutritional deficiencies caused by the enteropathy. Some CD patients require gluten-free supplements to address problems such as anaemia (iron, folate or vitamin B12) and loss of bone mineralisation (calcium and vitamin D) and coagulopathy (vitamin K). It may be necessary to provide lactose-free sources of calcium if secondary lactose intolerance, due to enteropathy-induced lactase insufficiency, remains unresolved.

The mainstay of treatment for patients with CD is lifelong maintenance of a gluten-free diet. This means avoiding all foods containing gluten in the form of wheat or related products (such as wheat, barley, rye, spelt, bulgar, durham, faro, couscous, semolina). Clear labelling is essential, along with an understanding of terminology for gluten-containing additives. Although it may seem obvious that foods made from wheat flour such as bakery products and pasta should be avoided, there are many foods and drinks containing gluten which may be less apparent. Such products include some seasonings, gravies, sauces, sausages and processed meats, salad dressings, chutneys and pickles, meat and fish pastes, blue cheeses, malt vinegar, malted or chocolate drinks, beers, whisky. A gluten-free lifestyle also means that some toothpastes, lipsticks and pharmaceutical products will also need to be avoided.

Thus, adhering to a gluten-free lifestyle is difficult and burdensome with a significant negative impact on quality of life. The introduction of a gluten-free diet requires support and encouragement to increase compliance and 'palatability'. In addition to the use of dietitians to advise newly diagnosed CD sufferers, information, recipes and cooking tips can be found from organisations aimed at supporting patients with CD such as the American Celiac Disease Alliance, Celiac Disease Foundation, Celiac.com, Celiac Sprue Association or Coeliac UK. The gluten-free diet consists of foods naturally gluten-free or those rendered gluten-free or almost gluten-free by processing. The maintenance of a completely gluten-free diet is

probably impossible. However, experts are unable to establish an agreed safe level of intake for patients with CD. Suggested levels have been put forward as between approximately 10 and 100 mg of gluten per day with <50 mg gluten per day being the aim for treatment of CD. The consensus amongst experts is that to manage CD, the gluten-free diet should be as strict as possible. To enable this, foods need to be labelled for gluten content, and in order to label foods as 'gluten-free' the term needs to be clearly defined.

A voluntary set of guidelines now exists in the European Union and in the USA. These are broadly similar between the regions with some differences in the details. These give definitions for a gluten labelling system to be voluntarily adopted by food producers. In the USA, the generation of the guidelines was facilitated by the *Food Allergen Labeling and Consumer Protection Act of 2004*, requiring that all food products manufactured after 1 January 2006 be clearly labelled to indicate the presence of the top allergens (milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, soybeans and wheat) (US FDA, 2004) 108–282, Title II). It also directed the Secretary of the Department of Health and Human Services to propose, and later make final, a rule that defines and permits the use of the food labelling term 'gluten-free'. As the list of allergens did not include barley or rye, the proposal for the labelling of gluten-free foods needed to define the term gluten, and the natural sources of gluten and related prolamins causing adverse responses in individuals with CD. Therefore the FDA proposed the following to define 'gluten-free' products for voluntary use in labelling of foods (Federal Register Proposed Rule – 72 FR 2795) (FDA, 2007). This was aimed to assist the maintenance of a gluten-free lifestyle in patients with CD. The FDA proposal defines gluten as proteins which occur naturally in 'prohibited grains' that may cause adverse health effects in persons with CD. These 'prohibited grains' are those from wheat (genus *Triticum*), rye (genus *Secale*), barley (genus *Hordeum*) or cross-bred hybrids of any of these grains (e.g. triticale, as cross between wheat and barley). The FDA proposes to define the term gluten-free to mean that the food does not contain any one of the following:

- (1) An ingredient that is a prohibited grain;
- (2) An ingredient that is derived from a prohibited grain and that has not been processed to remove gluten;
- (3) An ingredient that is derived from a prohibited grain and that has been processed to remove gluten, if the use of that ingredient results in the presence of 20 parts per million (ppm) or more gluten in the food; or
- (4) 20 ppm or more gluten.

Foods which fulfil these criteria of 'gluten-free' may then be labelled as such on a voluntary basis. For naturally gluten-free foods (with the exception of oats) the foods may be labelled as such provided that both the following criteria are met:

- (5) The wording of the claim clearly indicates that all foods of the same type, not just the brand bearing this labeling claim, are gluten-free (e.g., 'milk, a gluten-free food,' 'all milk is gluten-free'); and
- (6) The food does not contain 20 ppm or more gluten.

In Europe, most countries have accepted the definition of 'gluten-free' from that given by the CODEX Alimentarius Commission set up by Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) regarding foods for special dietary use for persons intolerant to gluten (CODEX, 1979). This set of guidelines again defines gluten and identifies the original dietary sources, but lists oats as discretionary and to be included in the gluten-containing foods, if wished, at a national level.

It stipulates that the level of gluten in foods labelled 'gluten-free' should not exceed 20 mg/kg gluten. A second category of foods labelled 'very low gluten', containing sources of gluten but processed to reduce the gluten content, must not contain more than 100 mg/kg gluten. The UK Food Standards Agency adopted guidelines based on this standard and that of the European Commission (EC, 2009) with definition of 'gluten-free' being up to 20 ppm and a 'very low gluten' category for foods containing not more than 100 ppm gluten. This system of labelling was instigated on 1 January 2012 with the introduction of a new law (Statutory Instrument, 2010) enforcing these standards of labelling for gluten content in all foods whether or not pre-packaged.

23.5.6 Future trends

Although currently the only treatment for CD is the maintenance of a gluten-free lifestyle, there are a number of novel approaches to managing CD under investigation.

The first group of strategies seek to reduce or remove gluten from the food. There have been a number of projects removing glutens from wheat using selective breeding programmes or genetic manipulation. To date, these have had limited success, probably due to the large number of different sequences coding for glutens contained within the wheat genome. A different approach to the removal of gluten is to use specific enzymes to pre-digest the food and reduce or remove gluten residues. Prolyl endopeptidases (PEP), brush border proteases which digest the resistant gluten peptides, have been isolated from micro-organisms (*Flavobacterium meningosepticum* or *Aspergillus niger*) or from germinating barley (endoprotease B2) and administered with a meal containing gluten. In animal models and *in vitro* the use of such peptidases has been shown to significantly reduce the immunogenicity of whole wheat gluten, e.g. ability to stimulate gluten-specific T cells (Gass *et al.*, 2006, 2007). There are also studies of the use of lactobacilli, which produce a range of proteases, in the production of sourdough bread in CD patients, again with some success (Di Cagno *et al.*, 2004). However, large amounts of the enzymes are required for a prolonged period to achieve significant degradation of resistant gluten fragments. It may be that these enzymes, which are suitable for oral therapy, may be used alongside a gluten-free lifestyle in times when the accidental ingestion of gluten may be higher (travelling or special social occasions).

There have also been a number of studies investigating ways to prevent gluten uptake, antigen processing or antigen presentation. The observation that CD

patients have an increased intestinal permeability led researchers to investigate the causative mechanism. An accidental discovery of a protein called zonulin was made whilst investigating the disrupted epithelial integrity caused by cholera (*Vibrio cholerae*) (Fasano *et al.*, 2000). Fasano and colleagues discovered that the upregulation of zonulin caused increased intestinal permeability by disrupting the tight junctions between epithelial cells. Exposure of *ex vivo* CD patient intestinal biopsies to gluten caused an upregulation of zonulin (Drago *et al.*, 2006). Therefore Fasano developed a zonulin inhibitor. The treatment of CD patients with this zonulin inhibitor in early trials has shown a reduction in symptoms and in the production of auto-antibodies in patients given the active drug when compared with the placebo (Kelly *et al.*, 2009). Other researchers have looked at an inhibitor of TTG with the intention of preventing the processing of gluten polypeptides by TTG and the resultant highly immunogenic complexes. However, there are concerns that the non-specific blocking of all TTG may have undesirable consequences. Further studies are investigating the administration of peptides to bind and block the HLA DQ2 sites preventing antigen presentation of the gluten. Investigation of immune manipulation has targeted the blocking of the action of cytokines thought to be dominant in the CD autoimmune response (e.g. IL-15, IFN- γ), or the use of immune peptides related to gluten to induce immune tolerance to gluten.

In addition to the development of new treatments, there is current, and needs to be further, investigation into strategies for primary prevention. More information is necessary on the true prevalence and natural history of CD along with investigation of precipitating factors. There is evidence that early infant feeding practices influence the manifestation of CD. Breastfeeding has been demonstrated to be protective against both the development and severity of CD. Thus infants breastfed for a longer duration are less likely to develop CD than those breast fed for shorter durations (FalthMagnusson *et al.*, 1996; Peters *et al.*, 2001; Ivarsson *et al.*, 2002). Furthermore, infants breastfed at the time of introduction of gluten to the infant diet also seem to be significantly less likely to develop CD (Ivarsson *et al.*, 2002; Akobeng *et al.*, 2006). Additionally, there is evidence that among patients with CD, those who were exclusively breastfed for six months or longer developed CD significantly later than CD patients who were not (D'Amico *et al.*, 2005). D'Amico and colleagues (2005) also showed that CD patients who had been exclusively breastfed for at least 6 months had lower rates of failure to thrive and short stature than other CD patients.

Weaning practices may also influence the manifestation of CD. It has been postulated that the low prevalence of CD in Denmark may be a consequence of a more delayed and gradual introduction of gluten to the infant diet (Weile and Krasilnikoff, 1992). The timing of introduction of gluten may be of importance with some suggestions that early (<3 months of age) and late (>7 months of age) introduction may increase the risk of developing CD (Norris *et al.*, 2005). Primary prevention is the ultimate solution to the problem and only further investigation into the identification of protective and precipitating lifestyle factors and further understanding of the mechanisms of CD will facilitate this aim.

23.6 Conclusion

Adverse immune responses to food have a high and increasing prevalence in the industrialised world. Although some have been described in the literature for centuries others have only been recognised in the last 60 years. The causative mechanisms are still being elucidated for these responses. The diagnosis of 'food allergy' has a highly significant personal and societal burden. For the individual, in addition to the associated morbidity, there is an enormous psychological burden. The adoption of a strict allergen-free lifestyle has an immense impact on community and social interactions and quality of life.

On a broader scale, the cost of diagnosis and management of food allergy to an already over-challenged National Health budget is significant. Sladkevicius *et al.* (2010) used a model to predict the cost of treating milk allergy in infants born in a single year in the UK. This used an estimate of the incidence of cow's milk allergy of 2.5% in a birth cohort of 734 000 babies (the UK live birth for 2006). They based their model on the management of 18 350 infants with new-onset cow's milk allergy, modelling the use of resources for consultations (general practitioners, clinics and hospitals) and the use of medications and specialised formulas. They predicted that the management of milk allergy in infants born in a single year in the UK would cost 25.6 million pounds. This is the prediction for a single type of food allergen in a single group of patients.

Taken together, this emphasises a great need for high-quality research into the mechanisms and natural history of these conditions. A recent report by the USA National Institute of Allergy and Infectious Diseases into the diagnosis and management of food allergy highlights the paucity of research evidence for many aspects of clinical decision making for food allergic disorders (Boyce *et al.*, 2010). A greater understanding of the cause of breakdown of immune tolerance and the ensuing immune mechanisms would facilitate the development of novel therapies. A greater consensus in diagnostic criteria is emerging, assisted by international panels of experts and the generation of public guideline documents (Boyce *et al.*, 2010). There is still a need to validate the methods for the use of new diagnostic tools and to evaluate their use in the management of food allergic disorders. More information is required to clarify precipitating factors. What differentiates the development of sensitisation alone (the generation of food allergen-specific IgE) from the development of full symptomatic IgE-mediated food allergy? What triggers an asymptomatic, enteropathy-free, gluten antibody seropositive individual into a patient with coeliac disease? What factors contribute to resolution of allergy in some individuals or the lack of resolution in others? Why are some allergens (e.g. those from peanut) more likely to induce lifelong allergy when compared with others (e.g. milk)? Is antenatal or early postnatal exposure to allergen a protective or precipitating factor for food allergy?

Whilst academic and clinical research seeks to address these and other questions the only current management strategy for adverse immune responses to food is the successful maintenance of an allergen-free lifestyle. It is hoped that in the

future other therapies and options may be available to individuals diagnosed with an adverse immune response to food.

23.7 References

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Plate III (Chapter 23) Detection of allergen-specific IgE can help confirm diagnosis and identify the triggering food in IgE-mediated food allergy. Specific IgE can be measured in a blood sample or by using the skin prick test. The skin prick test involves placing a small drop of allergen solution on the skin and gently breaking the skin below the drop. The allergen in the solution will make contact with any allergen-specific IgE bound to mast cells in the skin (image kindly provided by N. D. Diaper).

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Plate IV (Chapter 23) Detection of allergen-specific IgE using the skin prick test. The allergen in the solution will make contact with any allergen-specific IgE bound to mast cells in the skin. The allergen cross-links bound IgE causing mast cell degranulation, histamine release and a wheal and flare reaction. This can be read 15–30 minutes after administration and compared with a positive (histamine) and negative (saline) control response (image kindly provided by N. D. Diaper).

24

Early nutritional programming

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Abstract: In this chapter the ‘Developmental Origins of Health and Diseases (DOHaD)’ hypothesis is presented, providing a summary of the evidence to support the thesis that susceptibility to chronic degenerative diseases is programmed by exposures early in life. The chapter then looks in more detail at the evidence that this hypothesis also extends to immune function, with a review of the data to support the ‘early nutritional programming of human immune function’.

Key words: immune programming, Developmental Origins of Health and Disease (DOHaD), infant immune development.

24.1 Introduction

Substantial evidence supports the thesis that susceptibility to morbidity and mortality from chronic degenerative diseases may be programmed by nutritional deficiencies early in life. More recent research suggests that the development of the human immune system may also be vulnerable to early life insults, leading to later defects in immunocompetence. In this chapter, evidence will be presented to support the concept of early nutritional programming of human immune function, focusing on the following key themes:

- *Developmental Origins of Health and Disease (DOHaD) hypothesis:* This section will introduce the DOHaD hypothesis, presenting some of the original findings that led to the rapid expansion of work in this area. This section will also highlight the relevance of the hypothesis in developing country context where a high incidence of low birth weight is often coupled with a rapid nutrition transition and accompanying non-communicable disease profile. The assessment of maternal dietary intake will also be reviewed.

- *Programming of infectious disease mortality:* This section will introduce the evidence that mortality from infectious diseases may have an early life origin, and introduce the concept that immune development, and subsequent function, may be programmed by early-life nutritional insults.
- *Programming infant immune development:* This section will include an overview of the development of the infant's immune system, both pre- and early postnatally and include a review of the evidence supporting early life programming of infant immune development, with a specific focus on the thymus.
- *Programming long-term immune function:* This section will present the evidence supporting a long-term effect of early life insults on later immune function.
- *Future research directions and priorities:* The final section of this chapter will present the likely future direction of research in this field, including a review of where research is required to establish targeted public health interventions aimed at optimizing immune development.

24.2 Developmental origins of health and disease

Exposures during the prenatal and early postnatal periods are increasingly recognized as playing an important role in the aetiology of chronic non-communicable diseases (NCD), including coronary heart disease, stroke, hypertension, type 2 diabetes and osteoporosis. The 'Developmental Origins of Health and Disease' (DOHaD) hypothesis states that these disorders originate through unbalanced nutrition early in life. The growth of research in this field over the last three decades is largely attributed to the work of Professor David Barker and colleagues from the MRC's Environmental Epidemiology Unit in Southampton, UK. In the late 1980s, Barker and colleagues observed that differences in death rates from coronary heart disease in parts of England and Wales paralleled differences in neonatal and post-neonatal mortality in the early years of the 20th century (Barker and Osmond, 1986). Since most neonatal deaths were associated with low birth weight (LBW), these findings led to a series of studies of cardiovascular disease (CVD) risk in adults for whom archived birth records were available (Barker, 1994). These studies were able to demonstrate an inverse association between birth weight and a range of risk factors for CVDs (Barker, 2004), and were subsequently replicated many times from settings across the world, allowing a number of systematic reviews of the available data. For example, in an analysis including over 444 000 subjects from studies published between 1996 and 2000, Huxley *et al.* (2000) demonstrated that a 1 kg increase in birth weight was associated with approximately 2 mmHg decrease in systolic blood pressure. Although the evidence base has been criticized for publication bias and inappropriate statistical methods (Huxley *et al.*, 2002), the DOHaD hypothesis is now a major research area for multidisciplinary teams, focused on investigating possible biological mechanisms linking early life nutritional exposures and the pathogenesis of later disease.

The DOHaD hypothesis would predict highest risk in countries where an excess of infants are born below the WHO cut-off for LBW of 2.5 kg and where there is

a rapid transition to nutritional adequacy or excess in adulthood. Indeed, with increasing 'westernization', the health burden of NCDs in low- and middle-income countries continues to rise unabated, with CVD as the third leading cause of disability-adjusted life years and the leading cause of mortality (Miranda *et al.*, 2008). Many countries are therefore now facing a 'double burden' of disease, since this upsurge in NCDs is against a backdrop of a continued high risk of infectious diseases. Research focused on identifying the aetiology of both non-communicable and communicable disease in these settings – especially where the origins lie early in life – is therefore paramount to ensure that public health interventions can be targeted effectively.

The majority of published data within the DOHaD field use birth weight, or other anthropometric measures at birth such as ponderal index (mass/length³), as a proxy indicator of foetal nutrition. To ascertain causality, studies using more direct measures of maternal diet are desirable. Studies of nutritional deprivation, such as famine periods, have been used as a pseudo-experiment with which to investigate the long-term effects of maternal undernutrition. The most extensively studied example is the Dutch Hunger Winter of 1944. Between November 1944 and May 1945 there was a severe shortage of food in the western Netherlands as a result of German occupation and harsh weather conditions disrupting supply routes. Rations fell to between 400 and 800 calories a day during this period but were quickly restored with the Allied liberation in May 1945 (Roseboom *et al.*, 2006). However, since data from different Dutch Hunger Winter cohorts have been inconsistent (Painter *et al.*, 2005; Stein *et al.*, 2006), interpreting the effects of severe famine during pregnancy on long-term health effects is difficult.

A number of studies have collected detailed information on maternal dietary intake in relation to infant outcomes. Using data collected as part of the Avon Longitudinal Study of Parents and Children (ALSPAC), Leary *et al.* (2005) looked at blood pressure in 6944 children in relation to maternal daily nutrient intakes of carbohydrate, protein, fat, calcium, potassium and magnesium and also of the food groups milk, meat, fish, fruit and vegetables. The only finding was a weak inverse association with omega-3 fatty acids that was lost after adjustment for potential confounders, suggesting that diet in pregnancy in this setting does not influence offspring blood pressure. Indeed, in a review of some of the observational studies that have directly assessed diet during pregnancy on blood pressure as an outcome, Brion *et al.* (2008) concluded that there was no strong evidence that any component of the maternal diet influenced blood pressure in the offspring. However, data from the Cebu Longitudinal Health and Nutrition Study in the Philippines found that in Filipino adolescent boys, but not girls, low percentage energy from protein in the maternal diet was related to higher blood pressure at 16 years of age (Adair *et al.*, 2001), suggesting, possibly, that in less well-nourished populations the maternal diet in pregnancy may be more important for later programming of CVD risk factors such as blood pressure.

Taken together, the conflicting results emerging from observational studies of diet in pregnancy demonstrate the need for randomized controlled trials. Hawkesworth (2009), reviewed published trials to assess the impact of the prenatal

environment on CVD risk. Only a limited number of studies were found, focused on the follow-up of the offspring of women who participated in trials of either protein–energy, multimicronutrients or calcium supplementation trials. With the possible exception of multiple-micronutrients, there is little evidence to suggest that supplementation of pregnant women affects their offspring's CVD risk. However, this may be due to the limited amount of data currently available (number of trials, sample size, exposures investigated) coupled with the fact that none of the trials reviewed was set up for the purpose of investigating the impact of the maternal diet on later disease risk (Hawkesworth, 2009). Further trials, specifically designed to investigate the impact of the maternal diet on disease risk, are required.

24.3 Programming of infectious disease mortality

Evidence now exists to suggest that risk of infectious disease mortality may be 'programmed' during early life, possibly mediated by insults to immune development during infancy. The remainder of this chapter will therefore focus on the evidence to support the thesis that immune development may be sensitive to early life programming by nutritional exposures. Epidemiological data to support an early life effect on immune development primarily comes from the evidence linking intrauterine growth retardation (IUGR) to an increased risk of both morbidity and mortality from infectious diseases. In a review of the evidence, Ashworth (1998) demonstrates an increased risk of both diarrhoea and pneumonia in IUGR infants. More recently, and using pooled data from Nepal, India, Pakistan and Brazil, Black *et al.* (2008) showed that those infants born into the lowest birth weight group had a considerably greater risk of mortality from asphyxia, infections and all causes combined: for infectious causes, the relative risks were 4.2 (95% CI 1.5–11.7) for those weighing 1500–1999 g and 2.0 (95% CI 1.2–3.4) for those weighing 2000–2499 g, both compared with those weighing greater than 2500 g (reference group).

Twin studies have been an important component of studies investigating the DOHaD paradigm. Since birth weight often differs between twins, possibly reflecting a differential supply of nutrients to each twin, studies in twins offer an opportunity to examine the associations between birth weight and the offspring's disease risk independent of shared environmental or genetic factors (Villamor *et al.*, 2010). In a study examining the risk of tuberculosis in relation to size at birth among 21 596 Swedish twins born between 1926 and 1958, Villamor and colleagues demonstrated that the incidence of TB was inversely related to both birth weight and ponderal index: For every 500 g increase in birth weight, there was an 11% lower risk of TB, whilst every 0.2 g/cm³ increase in ponderal index was associated with an 8% lower risk (Villamor *et al.*, 2010).

Our own research conducted at the Medical Research Council's (UK) field station in Keneba, The Gambia, using the distinct pattern of seasonality as a model for looking at later health effects offers further support to this hypothesis. In The

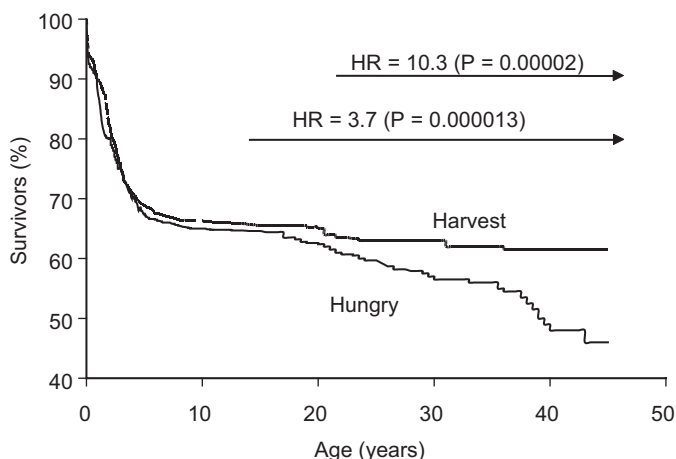


Fig. 24.1 Kaplan–Meier survival plots by season of birth. HR, hazard ratio. Adapted from Moore *et al.* (1999, Figure 2).

Gambia, there are two very distinct seasons. The annual dry or harvest season is characterized by low levels of active infections, good growth in infants and children, and a positive energy balance in adults as a consequence of abundant food from the previous year's harvest and limited agricultural work. In contrast, the annual wet or hungry season sees high levels of active infections and poor growth during infancy, and a negative energy balance is observed in all adults, including pregnant women. These distinct seasons result in seasonal patterns in many markers of health status, including pregnancy weight gains, gestational length and birth weight (Rayco-Solon *et al.*, 2005a,b). We know that these patterns are primarily nutritional in origin, since they can be reversed by maternal dietary supplementation (Ceesay *et al.*, 1997). Season of birth in this environment can therefore be used as a proxy marker for early life exposures in relation to subsequent health outcomes.

Using this seasonality combined with the demographic data for this population of rural Gambians dating back to the late 1940s, we looked at survival according to season of birth. Birth during the annual hungry or wet season led to a significantly greater risk of premature adult mortality compared with births during the dry season (Fig. 24.1) (Moore *et al.*, 1997, 1999). Since the majority of these adult deaths were infection related, this observation could suggest that an early life insult, correlated with season of birth, was permanently programming the developing immune system. Subsequent investigations in other settings with a pronounced seasonality, including Bangladesh (Moore *et al.*, 2004a), Senegal (Simondon *et al.*, 2004) and Burkina Faso (Kynast-Wolf *et al.*, 2006), coupled with demographic data have failed to replicate this observation, suggesting that the Gambian observation could be a highly discrete phenomenon localized in either time or place. Whilst the Gambian data should, therefore, be interpreted with caution, they support the thesis that the susceptibility to infectious disease mortality may also

have an aetiology early in life. The possibility that human immune function may be programmed by early life exposures to nutritional or other insults could have major implications for our understanding of disease processes in relation to infections. Understanding the nature of the early life insults and the biological mechanisms through which they operate is thus important.

24.4 Programming infant immune development

Many components of the human immune system develop early during foetal life and by birth all the systems are in place to allow the rapid maturity of the adaptive immune system towards immune competence. Although the immune system is qualitatively complete at birth, exposures during early life are critical for driving the adaptive expansion of the immune system. A developmental insult leading to the breakdown of any one of the complex pathways required to protect the host against pathogenic organisms could lead to an increased susceptibility to infectious disease.

To date, most attention within this field has focused on the thymus as a potential target for early nutritional programming. The thymus is a primary lymphoid organ essential for the establishment of a normal peripheral T-lymphocyte immune system. In the thymus, bone marrow-derived precursors undergo differentiation and selection, ultimately leading to the migration of positively selected thymocytes to the T-cell-dependent areas of the peripheral lymphoid organs (Savino, 2002, 2006). Thymic development is known to start early in the first trimester of pregnancy (Khlystova *et al.*, 2000), with the most critical period for thymic growth and development occurring between weeks seven and 14 of gestation (Adinolfi, 1997).

The description of the thymus as ‘a barometer of malnutrition’ came long before any role for the thymus gland in immunological responses was known (for a review, see Prentice (1999). In animal models, thymic growth is disproportionately affected by maternal undernutrition (Owens and Owens, 1989). and there is some evidence of the same phenomenon in humans (Naeye *et al.*, 1971). With such a central role in the early development of controlled adaptive immunity, and evidence that the thymus is vulnerable to early nutritional deficits, insults at critical periods of development may be critical for long-term function, and thus warrant investigation.

Postnatal thymic development can be assessed sonographically using a non-invasive validated method in which the transverse diameter of the thymus and the sagittal area of its largest lobe are multiplied to give a volume-related thymic index (Hasselbalch *et al.*, 1996b). This method has previously been used to indicate that the thymus is sensitive to early mode of feeding, with a smaller mean thymic index observed in bottle-fed than breast-fed infants (Hasselbalch *et al.*, 1996a). Ultrasound is therefore a useful tool to investigate the impact of the early life environment on infant thymic development.

In The Gambia, Collinson *et al.* (2003) assessed thymic development in a prospective cohort of 138 infants recruited prenatally and followed until 52 weeks

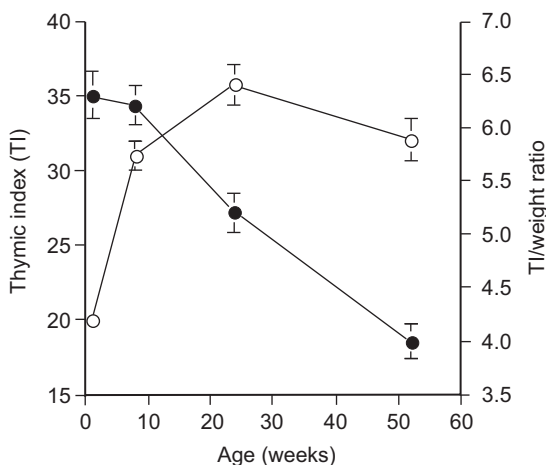


Fig. 24.2 Changes in mean thymic index (TI) with age (open circles) and in mean thymic index expressed in relation to body weight (TI/infant weight, kg) (filled circles). Adapted from Collinson *et al.* (2003, Figure 2).

of age. Thymic index was measured at one, eight, 24 and 52 weeks of age, together with the collection of growth and morbidity data. Figure 24.2 plots absolute thymic index and thymic index adjusted for body weight, against age at measurement. As illustrated, with respect to absolute size, mean thymic index increased dramatically up to 24 weeks of age, and then decreased to the measurement taken at 52 weeks of age. At all ages, thymic index was strongly associated with current weight and, in proportion to body size, mean thymic index was greatest at one week, decreasing to the 52-week measurement (Collinson *et al.*, 2003). This study also highlighted that an infant's thymic index tracks within individuals from birth to 12 months of age, even after adjustment for differences in body weight. At one and eight weeks of age, thymic index was associated with birth weight, but this did not persist after adjusting for current weight.

Of particular interest from this study was the observation of a strong effect of season on thymic size. Figures 24.3 (a) and (b) show the percentage difference in mean thymic index at each age between hungry/wet and harvest/dry season births (a) and hungry/wet and harvest/dry season measurements (b). Thymic index at week one was marginally lower in the hungry/wet season births, although this difference was not significant. Paradoxically, there was a trend towards a larger thymic index at 24 weeks among hungry/wet season births. There was no association of thymic index with birth season at eight weeks or 52 weeks, or for all ages combined. The corresponding analysis of mean thymic index according to season of measurement shows a consistently smaller mean thymic index in the hungry/wet season, which was significant at all time points, with the greatest difference seen at eight weeks of age.

To expand on these observations from The Gambia, infant thymic development has been assessed in a larger cohort of infants from Matlab, Bangladesh. Similarly

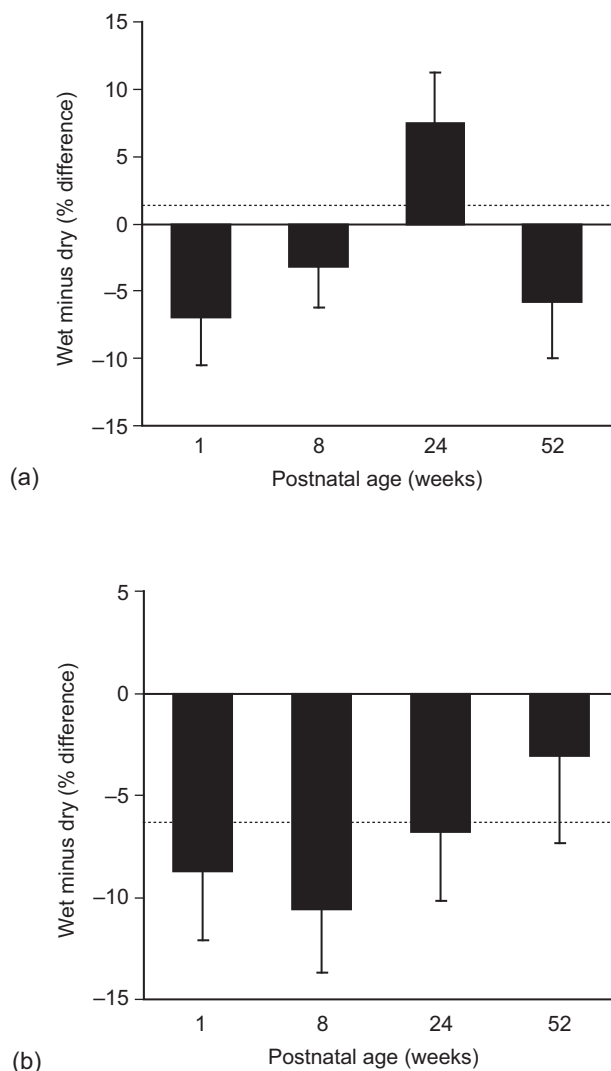


Fig. 24.3 Percentage (standard error) difference in mean thymic index (a) between hungry and harvest season births, and (b) between hungry and harvest season measurements, adjusted for gender, gestation and current weight. Effects of season on overall mean thymic index (dotted line) are also adjusted for postnatal age at each scan. Adapted from Collinson *et al.* (2003, Figure 3).

to The Gambia, life in rural Bangladesh is heavily influenced by an annual monsoon season (July to September). The detailed demographic data held in the Matlab Health and Demographic Surveillance System, together with the observed seasonality, provides an opportunity for comparisons with the data obtained from The Gambia.

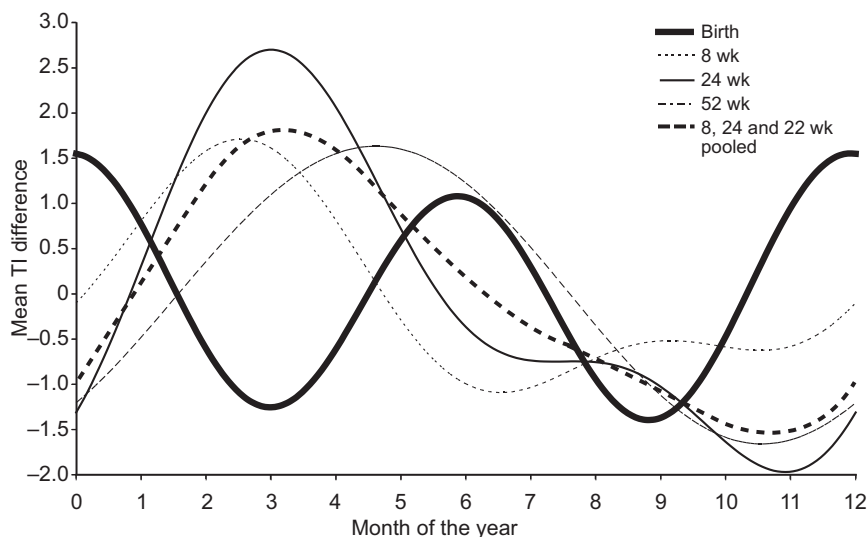


Fig. 24.4 Relative size of thymus variation with time of year plotted at birth and at 8, 24 and 52 weeks of age. Adapted from Moore *et al.* (2009, Figure 2).

Between 2000 and 2004, the International Centre for Diarrhoeal Disease Research, Bangladesh, conducted a large-scale study of combined interventions to improve maternal and infant health in Matlab – the ‘Maternal and Infant Nutrition Interventions, Matlab’ (MINIMat) trial. The study was designed to test community interventions aimed at improving birth outcomes and infant health, and included two nutritional interventions during pregnancy, and either counselling to promote exclusive breastfeeding or counselling on other health messages (Tofail *et al.*, 2008). The study design allowed an investigation of the effects of early life nutrition and environmental exposures on thymic development (Moore *et al.*, 2009).

Thymic size was assessed at birth (health centre deliveries only) and then at eight, 24 and 52 weeks of age. As with the data from The Gambia, thymic index at all ages was positively associated with infant weight. The effect of month of measurement on mean thymic index at each time point and for all time points pooled is illustrated in Fig. 24.4. Adjusted for sex and infant length at the time of measurement, a significant association with season of measurement was observed at all time points, with the exception of the measurements made at week eight (Moore *et al.*, 2009). These data provide further evidence that the thymus is sensitive to an environmental exposure correlated with month of measurement.

Thymic index at birth and at eight weeks correlated positively with birth weight, but by 24 and 52 weeks and when adjusted for infant weight this effect was no longer present. Using an intention to treat analysis, thymic size was not affected by pre-natal maternal supplementation, possibly reflecting the lack of any true control group. However, a longer duration of exclusive breastfeeding resulted in a larger thymic index at 52 weeks of age and was strongly correlated to arsenic exposure during pregnancy (Moore *et al.*, 2009).

These data from this much larger cohort of infants in Bangladesh support the previous observations from The Gambia and demonstrate that the development of the infant thymus is sensitive to certain nutritional and environmental stimuli. Of interest, in a study of Italian infants, IUGR was also associated with a disproportionately small thymus (Cromi *et al.*, 2009), suggesting that the effect is not restricted to populations where nutritional vulnerability is accompanied by a high infectious exposure. A limitation of all these studies, however, is the reliance on thymic size as an indicator for early immune development with no direct assessment of thymic function included. Studies exploring the long-term consequence of an impaired development of the thymus are therefore important.

Evidence exists from both animal studies and epidemiological research in humans to suggest poor thymic development may be a mechanistic link between IUGR and early mortality. Studies using rodent models show that low birthweight, resulting from maternal protein restriction during pregnancy followed by catch-up growth in rodents, is associated with shortened lifespan, whereas protein restriction and slow growth during lactation leads to an increased lifespan (Ozanne and Hales, 2004). It is proposed that these effects are mediated through differential thymic growth (Chen *et al.*, 2009). Data from infants in Guinea-Bissau, West Africa, indicate that a small thymus at birth (Aaby *et al.*, 2002) and six months of age (Garly *et al.*, 2008) is an independent risk factor for infection related mortality in infancy.

Little published data exists to correlate thymic size to function during infancy. In a small study of Danish infants, a positive correlation was observed between thymus size and the proportion of CD8⁺ cells in peripheral blood and the CD4/CD8 ratio at 10 months of age (Jeppesen *et al.*, 2004). With such a strong influence of season of measurement observed on thymic size in The Gambia, Collinson *et al.* (2008) also investigated seasonal influences on lymphocyte subpopulations in the same cohort of infants. Age-related changes were seen in all measured lymphocyte subpopulations, and successive subpopulation counts in individual infants were significantly correlated, suggesting an immune phenotype. Both cord and post-natal lymphocyte and leucocyte counts were substantially higher in the hungry/wet season than in the harvest/dry season births. Absolute CD3⁺, CD8⁺ and CD56⁺ counts were correspondingly higher in hungry season births, although CD4⁺ counts and CD19⁺ counts showed no significant association with birth season (Collinson *et al.*, 2008). Whilst not directly linked to thymic size, these findings suggest a seasonal influence on cell counts that is as pronounced in cord blood as it is postnatally.

One limitation in the direct assessment of thymic function is the absence of specific markers to assess recent thymic emigrants. Thymic output can be estimated, however, by using the frequency of signal-joint T-cell receptor-rearrangement excision circles (sjTRECs). These sjTREC represent the extrachromosomal excision products that are formed by the excision of DNA during recombination events in the genome to produce the α chain of the T-cell receptor in the thymus. The quantification of the frequency of sjTRECs levels is therefore an indirect measure of thymic output. Although there is still some debate around the validity of

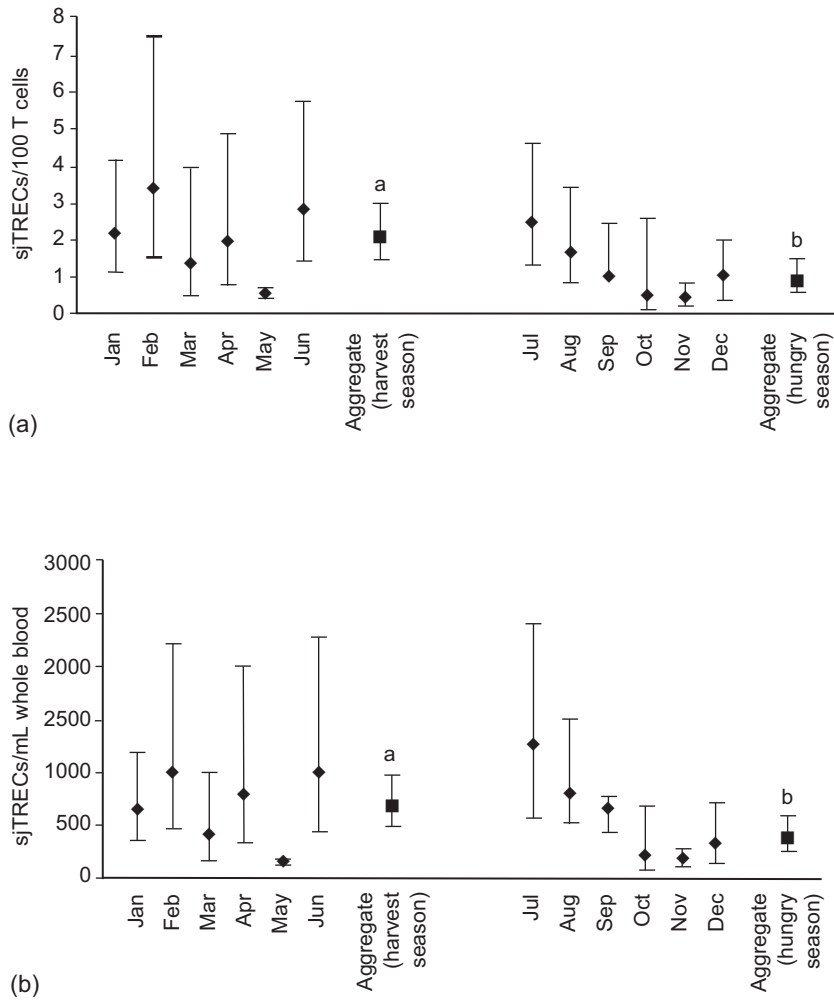


Fig. 24.5 The distribution of geometric mean signal-joint T cell receptor-rearrangement excision circle (sjTREC) concentrations in T cells (a) and whole blood (b) by month of birth in The Gambia. Aggregate seasonal geometric mean of sjTREC counts for the harvest (a) and hungry (b) seasons are shown as square points. Error bars represent 95% CIs, adapted from Ngom *et al.* (2004, Figure 1).

measures, they are currently the best available way to assess thymic function and immunosenescence and have been used to assess thymic output in a large number of studies.

To determine whether the observed difference in thymic size would be reflected by similar differences in sjTREC levels, blood samples collected from infants at eight weeks of age in The Gambia were assessed (Ngom *et al.*, 2004). Figure 24.5 illustrates the distribution of geometric mean sjTREC concentrations in T-cells and in whole blood by month of birth, with the aggregated mean for the harvest and

hungry seasons indicated. In spite of considerable monthly variation, a notable decline can be seen in the hungry/wet season, with the aggregated means being significantly lower than for the hungry/wet season infants. This finding could indicate that the seasonal effect on thymic size is reflected by a direct reduction in thymic output. As novel methods to assess thymic export are identified (Bains *et al.*, 2009; Haines *et al.*, 2009), further work is required to assess the longer-term impact of impaired thymic development in early life.

Much of the work within the DOHaD field points to the importance of both pre- and postnatal exposures in determining risk of later disease. In the study of Collinson *et al.* (2003) described previously, it is interesting that the observed season of measurement effect on thymic size was greatest when the infants were aged 8 weeks. Since in this community infants at this age are exclusively breastfed, are growing well and have minimal incidence of active infections, this observation could suggest the involvement of breastmilk factors on thymic development. In the Gambia, Ngom and colleagues (Ngom *et al.*, 2004) tested for the presence of a candidate breastmilk immune factor interleukin (IL)-7, a cytokine critical for thymic and T-cell development. Despite considerable monthly variation, breastmilk IL-7 concentrations were significantly lower in the hungry/wet season compared with the harvest/dry season: 79 pg/ml (95%CI 69.4–90.9) vs 100 pg/ml (95% CI 83.5–119.8), $p = 0.02$ (Ngom *et al.*, 2004). This observation suggests a putative role of breastmilk factors in thymic development, and requires further verification.

24.5 Programming long-term immune functions

Evidence to support a permanent effect of early life developmental insults to the immune system on long-term function is currently only available from cohort studies where immune function has been assessed in relation to early life exposures. Whilst most early studies within the DOHaD field focused on associations with risk factors for non-communicable disease, two studies from the Southampton group explored immune outcomes. In the first, Phillips *et al.* (1993) looked at susceptibility to autoimmune disease in a cohort of adult women, in relation to their size at birth and at one year of age. The proportion of women with thyroglobulin and thyroid peroxidase autoantibodies fell with increasing birth weight, although no association was found with weight at one year of age. In interpreting these data, the authors speculate that early growth restraint could affect immunological tolerance and the susceptibility to autoimmune disease, possibly mediated by impaired thymic development (Phillips *et al.*, 1993). In the second study, Godfrey *et al.* (1994) looked at serum levels of IgE among men and women aged 50 years in relation to head circumference at birth. Head circumference was found to be positively associated with IgE levels in adult life, leading to the suggestion that foetal undernutrition may programme individuals towards an allergic phenotype in later life, possibly mediated by impaired thymic maturation during a critical period in foetal development (Godfrey *et al.*, 1994). However, a subsequent study found no association between head circumference at birth, thymus size at one week of age

and allergic disease at five years of age in a cohort of Danish infants (Benn *et al.*, 2001).

One hypothesis to explain the link between early life exposures and later morbidity and mortality is that the observed immunocompetence results from an underlying defect in immunological memory, similar to the immunosenescence of old age. To test this hypothesis, in a study of 60 Gambian men (18–23 years) selected to represent births in the hungry/wet versus harvest/dry season, Ngom *et al.* (2011) looked at markers of thymic function and T-cell parameters. CD4⁺ and CD3⁺ but not CD8⁺ counts were lower for those born in the hungry/wet season and CD8⁺ telomere length also tended to be shorter, possibly suggesting an elevated risk of premature immunosenescence in this population exposed to likely early under nutrition (Ngom *et al.*, 2011). Such effects could also be reflected in the phenotype and kinetics of T-lymphocytes in subjects. To investigate this hypothesis, Ghattas *et al.* (2005, 2007) measured *in vivo* T-cell kinetics by stable isotope labelling of T-cell subsets combined with gas chromatography–mass spectrometry, T-cell phenotype (CD3, CD4, CD8, CD45RA and CD45RO expression), and frequency of sjTRECs in young Gambian men. No differences were observed in T-lymphocyte homeostasis in relation to markers of perinatal nutritional compromise (birth season and birth weight) (Ghattas *et al.*, 2007). However, it may be that differences in functional immunoresponsiveness would become apparent in the response to an immunological challenge.

One of the best *in vivo* methods to interrogate the immune system is to look at antibody response to vaccination and, because of the additional benefit vaccines yield to the study subjects, this is now a commonly used tool in epidemiological studies in this field. In a study of adolescents from the Philippines, McDade and colleagues showed that the predicted probability of mounting an adequate antibody response to a *Salmonella typhi* Vi vaccine was found to be lowest in adolescents who were born small for gestational age (SGA) and had the lowest body mass index at age 14–15 years (McDade *et al.*, 2001). Figure 24.6 shows the predicted probabilities of mounting a positive antibody response two weeks after vaccination for adolescents born SGA or appropriate for gestational age and with lower or higher BMIs at age 14–15 years. As illustrated, the best response was observed in those individuals who were born appropriate for gestational age and had a low BMI in adolescence, suggesting a combined effect of pre- and postnatal nutritional status.

These individuals were recently followed up to look at the association between vaccine responsiveness in adolescence (14–15 years) and C-reactive protein (CRP) levels in young adulthood (20–21 years) (McDade *et al.*, 2011). Median CRP concentration was more than four times higher among non-responders to vaccination than responders suggesting a within-individual link between more effective antibody-mediated immune defences and lower levels of inflammation. This data may indicate a long-term lasting effect of the early life environment on an individual's immuno-phenotype (McDade *et al.*, 2011).

Research from our own group had also demonstrated a positive correlation between birth weight and antibody response to the same Vi polysaccharide vaccine

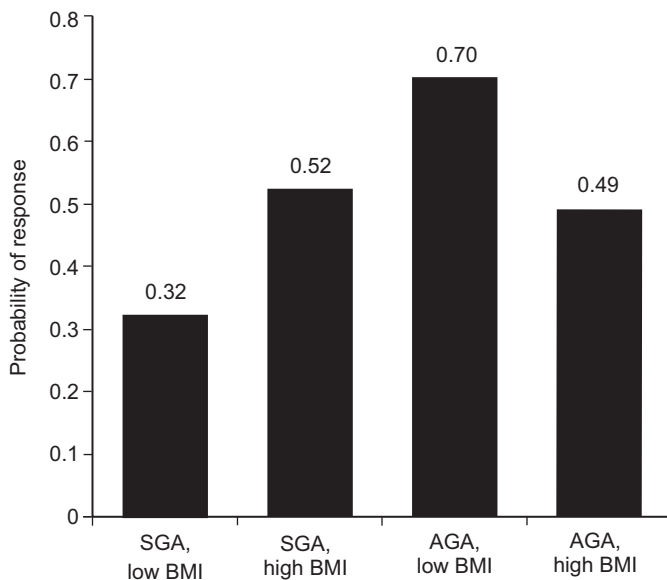


Fig. 24.6 Predicted probabilities of mounting a positive antibody response 2 weeks after vaccination for adolescents born small for gestational age (SGA) or appropriate for gestational age (AGA) and with lower or higher BMIs at age 14–15 years. Adapted from McDade *et al.* (2001, Figure 1).

in a cohort of adults born in an urban slum in Lahore, Pakistan (Moore *et al.*, 2004b). In the early 1960s an urban slum settlement in Lahore was selected for a community-based follow-up of infant health (Jalil *et al.*, 2008). Between 1964 and 1978, a total number 2468 infants were recruited into this study, and followed longitudinally into childhood. In 2002, a sub-group of 257 subjects were traced and recruited, selected consecutively on the basis of their living not too far from Lahore and their willingness to participate in the study (Moore *et al.*, 2004b). Each subject received the same *Salmonella typhi* Vi vaccine as given in the study of McDade *et al.* (2001) in the Philippines, and also two doses of rabies vaccine.

Figure 24.7 shows the mean birth weight according to vaccine response. For both the IgG and IgM antibody response, subjects showing a greater than four-fold increase in antibody titre were significantly heavier at birth. No association, however, was found between size at birth and the response to the rabies vaccine. It is generally accepted that true polysaccharide vaccines are T-cell-independent antigens and can induce an antibody response without the help of T-helper cells (Weller *et al.*, 2005). As a consequence, such vaccines are ineffective in young children when their B-cells are not yet mature. The contrasting effects of the two vaccines in this study from Lahore could, therefore, indicate that the mechanism for antibody generation to polysaccharide antigens is more susceptible to foetal growth retardation.

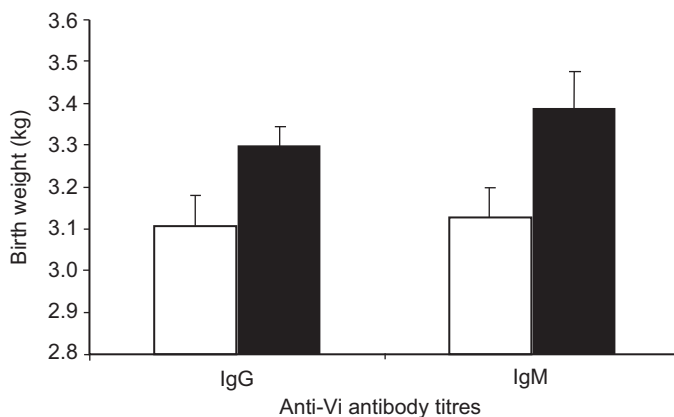


Fig. 24.7 Mean ($\pm$ SEM) birth weight by antibody response to typhoid vaccine in subjects showing a <4-fold increase in anti-Vi antibody titres (open bars) and in those showing a >4-fold increase (filled bars). Adapted from Moore *et al.* (2004b, Figure 2)

To investigate whether this deficit in response in the lower birth weight babies would be overcome by giving a booster dose of the vaccine, we revaccinated subjects with a second dose of the typhoid Vi vaccine (Moore *et al.*, 2008). In this second phase of the study, we additionally gave a polysaccharide conjugate vaccine (Hib) to see if the impaired response to polysaccharides would be observed in the conjugated vaccine. Anti-Vi IgG levels remained positively correlated with birth weight, but no associations were observed between anti-Hib IgG levels and size at birth (Moore *et al.*, 2008). Despite the use of different polysaccharides, the findings from this study would seem to suggest that the mechanism of response to a polysaccharide–protein conjugate vaccine is appropriately different to the response to a pure polysaccharide vaccine and that it is not sensitive to early life exposures. Further studies are required to understand the mechanisms linking early life nutrition to vaccine unresponsiveness in later life.

24.6 Future trends

The past three decades have seen a considerable expansion within the DOHaD field beyond examining the link between birth anthropometry and risk of subsequent CVD. Most notably, it now encompasses a more detailed range of exposures and a broader set of outcomes. Relevant to the current book is the evidence that human immune development may be sensitive to critical exposures early in life, although the evidence base at present is limited. Further studies are thus required to specifically try and identify the biological mechanisms involved in the early programming of immune function. Whilst prospective studies in newborns and cross-sectional studies in children and adults will help relate early exposures to later immune measures, to establish true causality and determine mechanisms for targeted intervention, randomized controlled trials of nutritional supplementation

during these critical periods in early life are required. Trials such as the Early Nutrition and Immune Development 'ENID' Trial in The Gambia (ISRCTN 49285450) aim to directly assess, through a randomized controlled design, the impact of combined pre- and postnatal nutritional supplementation on infant immune development. To help understand mechanisms, future research must also incorporate modern techniques for the assessment of immunity. Where conclusive evidence is presented to link early nutrition to later immune function, targeted intervention strategies may then be developed to help achieve a healthy immune system for all.

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630 Diet, immunity and inflammation

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25

The immune system during ageing

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Abstract: This chapter deals with the overall age-associated changes observed in human immunity, beginning with an overview of the components of the immune system. We then summarize the effects of age on these different components, discussing the problems faced by investigators studying ageing in long-lived outbred species like humans. We describe the limitations inherent in cross-sectional studies together with some possible solutions to these problems, such as longitudinal studies. We then discuss the possible reasons for these age-associated changes, starting with hematopoiesis and the effects of age on stem cells and continuing with the phenomenon of thymic involution. We consider biomarkers termed ‘immune signatures’ and those forming an ‘immune risk profile’. We conclude with a brief consideration of overall impact of age on responses to vaccination in humans.

Key words: immunosenescence, immune risk profile, inflammaging, immunogerontology, immune frailty.

25.1 The components of the immune system and their origins: innate and adaptive, cellular and humoral

25.1.1 Introduction

The role of the vertebrate immune system is to defend the body against pathogenic agents. The first line of defence is the skin and the mucosa of the respiratory, gastrointestinal and urogenital tracts, which provide physical and physiological barriers. These barriers (see Fig. 25.1) provide physical resistance but also involve immune functions and other active defences, also called innate immunity; how

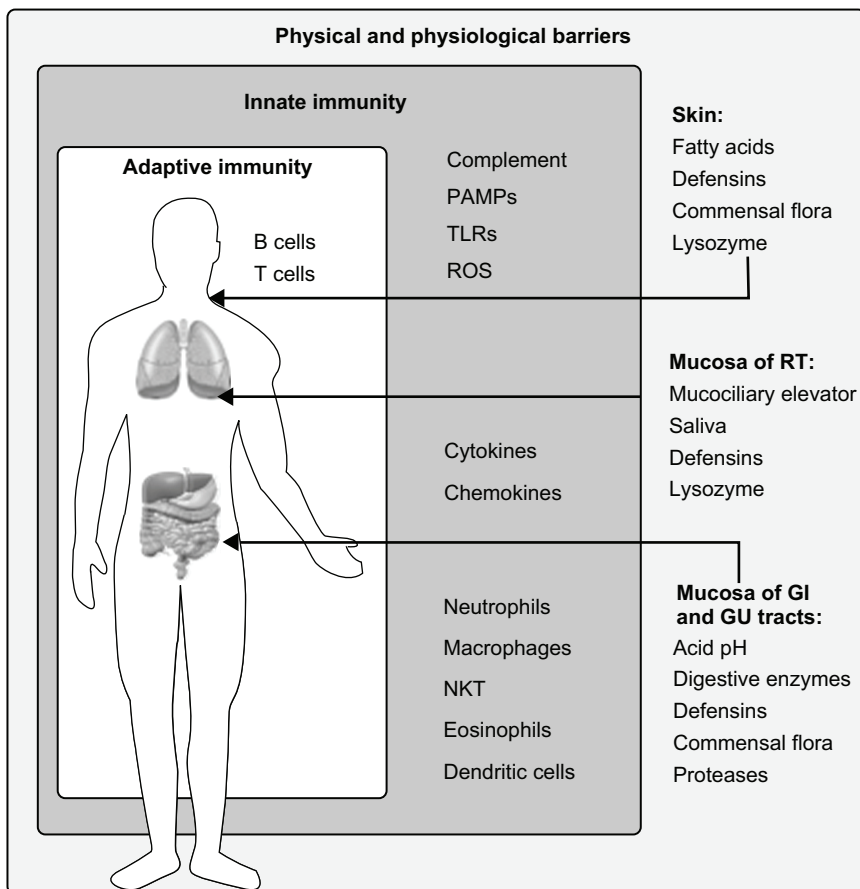


Fig. 25.1 Overview of physical and physiological barriers and components of the immune system. GI, gastrointestinal tract; GU, genitourinary tract; NKT, natural killer T cells; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species; RT, respiratory tract; TLRs, Toll-like receptors.

ever, they are never perfectly protective. When a pathogen breaches these barriers, the evolutionarily ancient innate immune system provides an immediate response commonly involving phagocytic activity and local release of toxic agents. Specialized receptors on the surface of innate immune cells recognize structures common to pathogens, but absent from vertebrates, the so-called 'PAMPs' (pathogen-associated molecular patterns). Invertebrates possess only this type of cellular immunity. However, for reasons not entirely clear, vertebrates have invested very significant resources in generating and maintaining a whole second immune system, designated 'adaptive immunity', which relies on monospecific immune cells, recognizing one single antigen, but with the ability to 'remember' the antigen they have encountered. Thus, one hallmark of the adaptive immune response is its ability to produce memory T cells after the infection is cleared. This allows a more

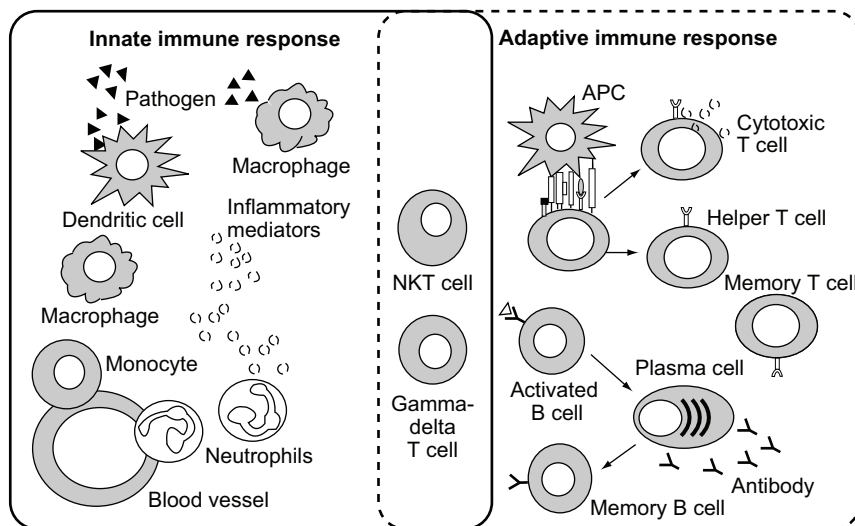


Fig. 25.2 Interplay between components of innate and adaptive immune responses. APC, antigen presenting cells; NKT cells, natural killer T cells.

rapid and efficient clearance of the pathogen in case of a reinfection (Rollinghoff, 1997), a feature not shared with the innate immune system to any great degree. Recently, however, the distinction between innate and adaptive immunity has become blurred, with innate immune cells demonstrating certain memory characteristics and antigen-specific B cells and memory T cells expressing receptors characteristic of innate cells, making a strict binary classification less obvious (Lanier and Sun, 2009).

25.1.2 Characteristics of innate and adaptive immunity

Innate immune mechanisms (see Figs 25.1 and 25.2) can be found in representative species at almost every level of evolution (Cooper and Herrin, 2010), illustrating the importance of innate immunity, even though by definition this system does not generally confer improved immunity on re-exposure to the same pathogen. Innate immune defences are not non-specific, but they respond to pathogens in a generic way (Litman *et al.*, 2005).

Many different systems are employed in host defence (see Fig. 25.1). Innate immunity employs germline-encoded pattern recognition receptors for pathogens or damaged self-components which may mimic these. Other major characteristics include activation of the complement cascade and chemical humoral factors, recruitment of neutrophils, macrophages, natural killer cells (NK), eosinophils, and dendritic cells (DCs) to sites of infection and activation of the adaptive immune system. Most or all of these activities can be described as pro-inflammatory responses.

The main function of these responses is the elimination of the pathogenic damage that enables inflamed tissues to return to homeostasis. Initially, inflammation is characterized by an increase of blood flow to the site of infection or injury, increased vascular permeability and selective influx, and accumulation of different effector cells, such as neutrophils, monocytes and macrophages (see Fig. 25.2, left), which cooperate during the onset, progression and resolution of inflammation (Soehnlein and Lindbom, 2010). The regulated expression of adhesion molecules, chemokine receptors and other important molecules, as well as their counter-receptors on the surface of the inflammatory and endothelial cells, contributes to precise trafficking of leukocytes to the inflammatory site. Inflammatory mediators, facilitating this highly efficient process, include the coagulation system and neuropeptides, antimicrobial peptides, pro-inflammatory cytokines and chemokines, in addition to components of the complement cascade. At sites of inflammation, many different cell types, including neutrophils, macrophages and epithelial cells, express NADPH oxidase, which generates ROS (reactive oxygen species) playing an important role in inflammation (Fialkow *et al.*, 2007).

In addition to mediating strong antimicrobial activity (and causing extensive tissue damage as a side-effect), they also act as signal transduction molecules, contributing to the induction of a variety of different inflammatory cytokines, chemokines, adhesion molecules and enzymes (Yamamoto *et al.*, 2008; Soehnlein and Lindbom, 2010). The regulation of inflammatory reactions is then balanced by the interplay of pro-inflammatory cytokines, such as interferon (IFN)- γ , interleukin (IL)-1, IL-6, tumour necrosis factor (TNF), tumour growth factor (TGF)- β , IL-11, IL-12, IL-17, and anti-inflammatory cytokines, such as IL-4, IL-10, IL-13. The termination of the inflammatory response involves phagocyte-derived signals abrogating the recruitment of neutrophils and the clearance of apoptotic neutrophils, which leads to promotion of an anti-inflammatory programme and to the reconstruction of tissue homeostasis (Serhan *et al.*, 2008; Soehnlein and Lindbom, 2010).

Adaptive immune defences (see Figs 25.1 and 25.2, right) emerged around 500 million years ago with the appearance of clonotypic immunity in vertebrates. A defining feature of adaptive immunity is the somatic development by genetic rearrangements of the inherited germline of a pool of clonally diverse lymphocytes with a large repertoire of different antigen-recognition receptors. When these receptors bind their ligands under appropriate conditions, cell activation, clonal expansion and differentiation from naïve cells (i.e. cells that have not yet encountered antigens) to effector and memory cells is triggered. This type of combinatorial generation by rearrangement of a relatively limited number of discrete genetic components results in the generation of a highly diverse lymphocyte antigen receptor repertoire and allows the recognition of an almost infinite number of potential antigens and hence elicitation of highly specific responses to them (Litman *et al.*, 2010). The process of clonal expansion (to generate sufficient cells to combat the pathogen), differentiation to effector cells (to eliminate the pathogen), and finally retention of a small fraction of antigen-specific cells as memory cells (to respond faster and more strongly to pathogen re-exposure) are the hallmarks of adaptive immunity.

Adaptive immunity depends on B lymphocytes which produce antibody (humoral immune response) and T lymphocytes, which mediate cytotoxicity, secrete pro- and anti-inflammatory cytokines, and provide help for B cells (see Fig. 25.2, right). Immature B cells are continuously formed in the bone marrow, exported to the periphery, and differentiate to antibody-producing cells when appropriately activated by antigen and helped by T cells. Several steps of somatic recombination at the receptor (antibody) loci lead to production of a unique variable domain in the immunoglobulin functioning as the antigen receptor of each individual B cell. Millions of different clonotypes of B cells circulate continuously in the blood and lymphatic system performing immune surveillance. Upon direct recognition of foreign antigen and on receiving additional signals from a helper T cell, they differentiate into plasma cells and produce antibodies specific for the target antigen, and may further differentiate into memory B cells (see Fig. 25.2, right).

T-cell progenitors also originate in the bone marrow (see Fig. 25.3) from haematopoietic stem cells but, after export to the periphery, they must migrate to the thymus, giving rise to a large population of immature thymocytes in young individuals (Schwarz and Bhandoola, 2006). Initially, these only express the antigen-specific T-cell receptor (TCR), and are CD4- and CD8-double-negative. Upon further development in the thymus, most rearrange α - and β -TCR genes and express both CD4 and CD8 before further differentiation into single-positive CD4 (major histocompatibility complex, MHC, class II-restricted) or CD8 (class I-restricted) T cells, which are then released from the thymus. In order to prevent T cells from recognizing self-antigens and to ensure recognition of a variety of different pathogens, the thymocytes undergo the process of negative selection (if they bind strongly to self MHC) and then positive selection (for binding weakly to self MHC in the thymus) (see Fig. 25.3). The exported CD4 cells are characterized mainly as helper T cells providing help for B cells as well as for CD8⁺ T cells, but may also include T-regulatory cells (Tregs), which inhibit responses of other T cells. Most CD8 T cells are cytotoxic and are involved in lysis of infected cells. T cells are distinguished according to the nature of their TCR, which is composed of an α - and β -chain (in 95% of T cells, as described above) or a γ - and δ -chain with markedly different antigenic specificity and commonly remaining CD4 and CD8 double-negative (Rollinghoff, 1997). These latter cells are not MHC-restricted and commonly recognize lipid antigens.

Outside the thymus, T-cell activation occurs through binding of the TCR to its cognate peptide–MHC complex presented on specialized antigen presenting cells (APCs), particularly dendritic cells (Fig. 25.2, right). The TCR is present as a complex in association with the multichain CD3 moiety, the gamma, delta, epsilon and zeta chains of which act to couple the TCR to the signal transduction pathway required for T-cell activation. In addition to the signal from their receptor, T cells require co-stimulatory signals, which are delivered through monomorphic so-called co-stimulatory receptors, of which CD27 and CD28 are two major examples. When appropriate signals are present, the T cells become fully activated and embark on a programme of clonal expansion and differentiation, giving rise to a large pool of effector cells. In the absence of a co-stimulatory signal, however,

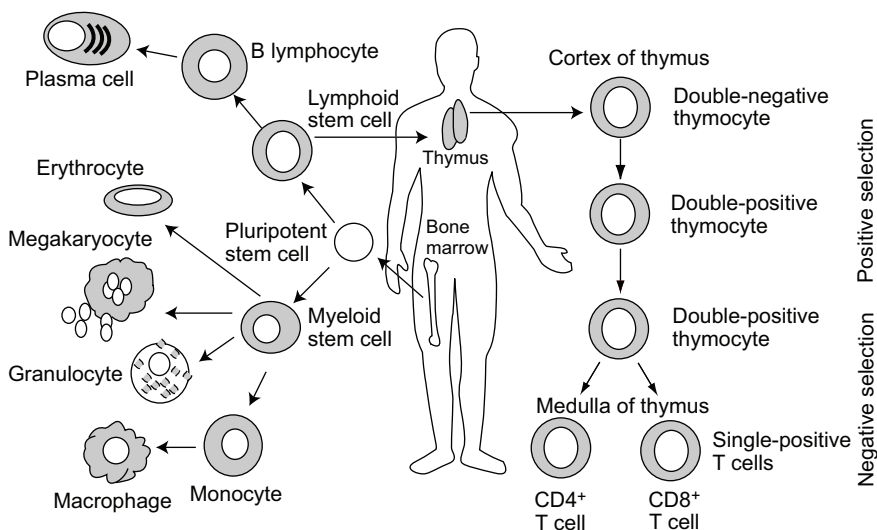


Fig. 25.3 Overview of steps of haematopoiesis (left) and thymopoiesis (right).

ligation of the TCR leads to inactivation of the T cell and development of a state termed anergy, thought to be important in tolerance.

25.2 The effects of age on the different components of the immune system

Ageing is a multifactorial process affecting all aspects of human biology, including immunity. The effects of ageing on the immune system are manifold (see Fig. 25.4) ranging from changes in haematopoietic stem cell status and generation of lymphoid progenitors in the bone marrow, results of thymic involution and differences in naïve and mature lymphocytes in secondary lymphoid organs. Together, these changes result in dysfunctional immunity in the elderly (Linton and Dorshkind, 2004).

Perhaps the most clinically significant of these changes is the decreased competence of the adaptive immune system reflected in the increased incidence and severity of infectious disease, increased incidence of myeloid diseases including leukaemias, and the onset of anaemia in the elderly (Linton and Dorshkind, 2004; Hock, 2010). The deleterious age-associated changes to immunity observed in all mammals studied so far are loosely bundled together in the term 'immunosenescence', affecting innate as well as adaptive immunity. However, the clinical impact of these changes is not clear, and exact mechanisms of, and biomarkers for, immunosenescence remain controversial (Derhovanessian *et al.*, 2009). Despite clear evidence that many immunological values are markedly different in old and young people, in most cases it is not clear whether these differences contribute to or even reflect poorer health status in the elderly. Thus, it

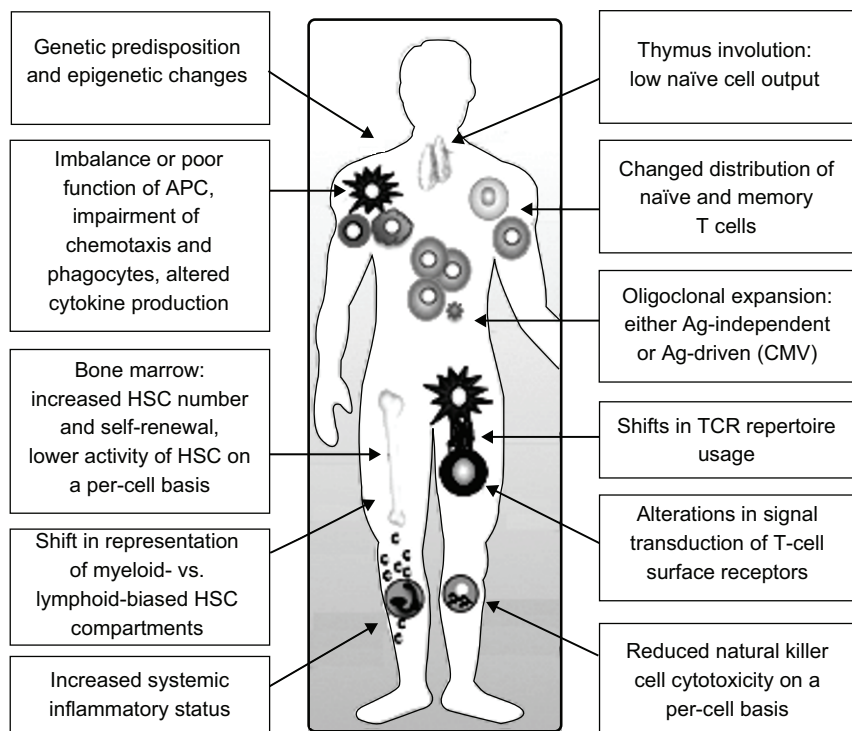


Fig. 25.4 Overview of effects of age on the different components of the immune system. Ag, antigen; APC, antigen presenting cells; CMV, cytomegalovirus; HSC, haematopoietic stem cell; TCR, T-cell receptor.

is predominantly circumstantial evidence that suggests that retained ‘youthful’ values of both innate and acquired immunity in the elderly actually does correlate with health status (Caruso *et al.*, 2009).

Another school of thought has it that the constellations of immune parameters measured in the elderly and found different to the young reflect the results of adaptation to the pathogen environment over a lifetime of pathogen exposures. This results in some functions being reduced when not required, but retained or increased. This also explains consensus findings that age-alterations are more noticeable in the adaptive immune system and that innate immunity is relatively well preserved (Sanson *et al.*, 2008). The ‘remodelling hypothesis’ can thus be interpreted in terms of adaptional and compensatory mechanisms (Pawelec, 2012). Such changes may be common denominators in ageing immune systems.

Thus, a consensus view of the essential features of immunosenescence at different levels of importance encompasses reduced NK cytotoxicity on a per-cell basis; reduced number and function of DCs in blood and Langerhans cells in skin; decreased numbers and diversity of naïve T and B cells; and increased memory and effector T and B cells. In particular, an accumulation of late differentiated effector

T cells, commonly associated with cytomegalovirus (CMV) infection (Derhovanessian *et al.*, 2009), likely contributes to a decline in the capacity of the adaptive immune system to respond to novel antigens (Grubeck-Loebenstein *et al.*, 2009).

Earlier studies of the behaviour of immune cells *ex vivo* and in culture *in vitro* have helped to generate models reflecting the perceived age-associated changes in human T cell-mediated immunity. However, exactly which factors are most important in immunosenescence and what causes these age-associated changes remain largely unclear (Derhovanessian *et al.*, 2008). There is likely to be individual variation in genetic predisposition in addition to the epigenetic changes over lifespan (see Fig. 25.4). A major confounding factor in human studies is how far immune dysfunction is a cause or an effect of the observed (or unknown) circumstances and history of each individual subject.

Key questions might be better addressed by longitudinal studies rather than the more common and logistically less demanding cross-sectional studies, but there are very few data on immunity available from the former at present. Many age-associated differences between young and old people have been reported, but whether these are actually changes or only differences has mostly been imputed not demonstrated. Additionally, correlations with health and longevity have likewise mostly only been hypothesized and not actually tested, because this would require follow-up studies and not the more usual cross-sectional approaches. Clearly, young and old groups studied at the same time will differ in many ways that are hard to define and control for, such as pathogen exposure, developmental and nutritional factors, population genetics, etc. (Pawelec *et al.*, 2010; Pawelec and Derhovanessian, 2011). Data from increasing numbers of challenging longitudinal studies are now beginning to emerge, bringing some surprises with them. One of the earliest of these studied populations >85 years of age, allows mortality follow-up at two, four and six years within a realistic investigational time frame (Wikby *et al.*, 1994).

25.2.1 Data gleaned from cross-sectional studies and their drawbacks

As mentioned above, cross-sectional studies comparing those currently young with those currently old reveal a large number of potentially age-associated differences, including immune parameters, such as the distribution of sub-populations of naïve and memory T cells as well as their functional integrity. Differentiation-associated markers influencing homing, co-stimulation, adhesion, etc., as well as differential expression of transcription factors affecting T-cell lineages, are routinely used to define ‘subsets’ of T cells. These are actually differentiation stages, continuous variables, which can also lead to confusion in the field.

One early model, still commonly used, defines naïve T cells as co-expressing CC-chemokine receptor 7 (CCR7) together with leukocyte common antigen (LCA) isoform CD45RA (Sallusto *et al.*, 1999). These cells possess long telomeres because they have not yet undergone repetitive rounds of cell division required for clonal expansion after activation (Rufer *et al.*, 1998). In contrast, memory T cells have shorter telomeres and express the LCA alternative splice variant CD45RO

but not CCR7 (Weng *et al.*, 1997a,b). As T cells approach a putatively senescent state, which may perhaps more conservatively be referred to as 'very late-stage differentiated', such characteristics as surface marker expression alter. The problem resides in distinguishing between adaptive differentiation pathways and 'ageing' processes, implying dysfunctionality. Thus, it is often stated that one of the hallmarks of immunosenescence is that aged T cells do not display CD28, a molecule critical for signal transduction and T-cell activation, leading to accumulation of these T cells. CD8⁺CD28⁻ T cells are absent in the newborn and become the majority of circulating CD8⁺ T cells in the elderly.

The accumulation of CD8⁺CD28⁻ T cells is also seen under other conditions, most often chronic antigen exposure. CD8⁺CD28⁻ T cells are derived from CD8⁺CD28⁺ T cells after repeated antigenic stimulation or persistent latent infection (Almanzar *et al.*, 2005; Nikolich-Zugich, 2008). In viral infections, this is especially the case for CMV, which is essentially ubiquitous in the elderly. Loss of CD28 is therefore likely to reflect a stage in the differentiation of CD8 cells from naïve to memory cells, and not a marker of 'senescence', and is especially markedly driven by CMV.

Altered distributions of naïve and memory T cells in peripheral blood of the elderly are commonly reported for CD8 T cells and to a lesser degree also for CD4 cells. In newborns, the ratio of naïve to memory T cells is high; in adults this ratio is reversed. The causes of age-associated decline in the generation of naïve cells are likely to be multifactorial and to involve progressive involution of the thymus, changes in growth factors and/or hormones, haematopoietic progenitor cells, and their surrounding microenvironment (see below). It is also because most of the naïve T cells have been exposed to antigen and hence converted to memory cells as a result of pathogen challenge during the lifetime. The accumulation of memory cells with age may also reflect an adaptive response to the decline of production of naïve lymphocytes through homeostatic cytokine-driven peripheral expansion, as well as the cumulative effect of past and persistent viral infections (Pawelec *et al.*, 2006; Weng, 2006). In the quite rare individuals who remain CMV-seronegative even at advanced age, this loss of naïve CD8⁺ T cells is much less marked (Chidrawar *et al.*, 2009; Derhovanessian *et al.*, 2010). Accordingly, several reports concur that immune status is markedly influenced by infection with CMV, which is not seen with other common and persistent herpes viruses (Derhovanessian *et al.*, 2009; Pawelec *et al.*, 2009; Pawelec and Derhovanessian, 2010). For whatever reason, a large proportion of available resources is required for CMV immune surveillance, which tends to increase with age. This may itself cause pathology due to the associated higher systemic levels of inflammatory mediators (Pawelec *et al.*, 2010). This results in an increased systemic inflammatory status, which might be of benefit for survival in infectious environments. However, as more people live longer lives, diseases associated with low-grade chronic inflammation, such as cardiovascular disease, diabetes and dementias are increasing, possibly the result of collateral damage, as it were. Those individuals predisposed in some way to 'resist' the effects of this increased pro-inflammatory status may be the long-term survivors in the population. There must, therefore, always be a compromise

between immunity required for surviving pathogen onslaught, and the unavoidable concomitant negative effects of inflammation.

Low relative levels of naïve T cells, especially CD8 cells, remains the most commonly reported and widely accepted biomarker of immune ageing, usually coupled with what is viewed as a reciprocal increase in memory cells with a range of different phenotypes determined by expression of co-stimulatory receptors CD27 and CD28 (Romero *et al.*, 2007). The latter generally manifest age-related TCR diversity contraction and cytokine secretion patterns. A large TCR repertoire is generally considered essential for maintaining adequate immune competence in the face of new pathogens, so diversity decreases with age are assumed to have negative implications. Repertoire contraction in CD4 cells may occur quite suddenly: TCR diversity has been reported to be well maintained up to an age of around 60–65 years, but is thereafter found to be precipitously reduced (Naylor *et al.*, 2005). This may well contribute to the poor responses to novel infections and to vaccination in this age group.

Other changes in T-cell populations, including increased presence of regulatory T cells, accumulations of oligoclonally expanded potentially functionally incompetent T cells, changes in the cytokine profile of T cells (not only due to altered frequencies of different T-cell subsets, but also to differential expression of co-stimulatory receptors and alterations in signal transduction pathways), decrease in the cytotoxic activity of NK T cells and shifts in TCR repertoire usage, can negatively impact immune responses in older individuals (Goronzy and Weyand, 2005; Weng, 2006). Failure to take these different frequencies of cell types into account confounds interpretations of functional differences between young and old.

In addition to the age-related alterations in the proportions of T-cell subsets, there are also changes at the level of the individual T cell. Alterations in numbers and efficacy of signal transduction of T-cell surface receptors, both antigen-specific and co-stimulatory, have been reported. These differences are partly due to the transit of molecular components through the cell membrane and hence altered formation of the 'immunological synapse'. The TCR remains unchanged in lymphocytes from the elderly, but assembly into these functional units ('signalosomes') is compromised. This is because cell membrane composition and resulting changes to fluidity (Rivnay *et al.*, 1980) affect TCR mobility and signalosome assembly. This is a prime example of a confounding factor, which is difficult to take into account in ageing studies, of central relevance to the theme of this book, namely, the impact of nutritional variation on immune parameters. Membrane fluidity is acutely altered by systemic fatty acid availability, and elderly people are almost certain to differ from the young in nutrition, whereby this marked effect of the fatty acid environment may be very important, as dramatically demonstrated by the finding of rapid alterations to T-cell membrane lipid composition, coupled with markedly decreased activation and proliferation potential following the infusion of lipids into young volunteers (Larbi *et al.*, 2005). Hence, the increased levels of cholesterol often found in older adults are likely to contribute to the age-associated decreases in T-cell activation (Larbi *et al.*, 2005).

Age-associated changes to B cells and their subsets are less well studied, but reports suggest that, like T cells, memory B cells accumulate with age in terms of proportions and numbers of CD27⁺ cells (Colonna-Romano *et al.*, 2003; Veneri *et al.*, 2009). However, other studies have provided inconsistent data (Breitbart *et al.*, 2002; Chong *et al.*, 2005; Frasca *et al.*, 2005; Shi *et al.*, 2005). One reason for discrepancies might be in the use of CD27-positivity only as identifying memory cells; the CD27⁺ memory subset is probably heterogeneous, comprising less differentiated memory cells with the phenotype CD19⁺IgM⁺IgD⁺CD27⁺ and the more differentiated 'switched' memory cells. The former decline with age in some studies (Shi *et al.*, 2005) but, again, others show no significant difference between young and old (Frasca *et al.*, 2008). IgD⁺CD27⁺ cells reciprocally seem to increase to the detriment of the IgD⁺CD27⁺ cells, but only in proportion and not in absolute numbers (Shi *et al.*, 2005; Frasca *et al.*, 2008). On the other hand, 'switched' IgD⁻CD27⁺ B cells decline in both absolute numbers and proportions in the elderly (Shi *et al.*, 2005; Frasca *et al.*, 2008). There also seem to be age-associated differences in antibody formation via altered capacities for class switching and somatic hypermutation, affecting the humoral response in the elderly (Frasca *et al.*, 2005). In addition, ageing is associated with the incidence of B-cell malignancy in older adults with oligoclonally expanded B-cells (Weng, 2006). Further data on B-cell alterations are needed, particularly since decreased absolute numbers of B cells were a component of the original immune risk profile (IRP) predicting two-, four- and six-year mortality in very elderly Swedes (see below).

25.2.2 Data from longitudinal studies pointing to clinically relevant changes with age

As alluded to above, longitudinal studies allow individual characteristics to be associated with health outcomes. Obviously, such studies are challenging in long-lived species like humans (Pawelec and Derhovanessian, 2010). The early Swedish OCTO/NONA studies focusing on the very elderly (>85 years) gave rise to the concept of an IRP (a cluster of immune parameters predicting mortality at two, four and six-year follow-up). Iterations of the IRP indicate that the most important characteristic is an inverted CD4/CD8 ratio. This is caused by expansions of late-stage differentiated CD8⁺CD28⁻ cells but not by contractions of naïve CD8⁺ T cells (instead, fewer B cells were part of the cluster). The OCTO immune study was performed on subjects selected for very good health at baseline and was the origin of IRP, indicating that a combination of high absolute numbers of CD8 cells and low CD4 cells, and poor mitogen-stimulated T-cell proliferation, together with low B cells, was associated with a higher two-year mortality (Wikby *et al.*, 1994). This was extended to include accumulation of the CD28⁺CD28⁻ CD8 cells and designated the 'immune risk profile' (Pawelec *et al.*, 2001). In follow-up studies, the IRP was also shown to be representative of general populations (i.e. not only those selected for exceptional health) (Wikby *et al.*, 2006). Moreover, inflammatory markers, such as IL-6, C-reactive protein, transthyretin and albumin, were also, independently, predictive of mortality.

These findings suggested to us that a sequence of events beginning with CMV infection earlier in life results in the generation over time of ever-larger numbers of CD8⁺CD28⁻ cells to control persistent viral infection, and eventually the development of an IRP associated with elevated inflammatory status [(100% of IRP⁺ individuals are CMV-seropositive (Wikby *et al.*, 1998; Olsson *et al.*, 2000)]. Later data indicated that becoming a centenarian was associated with never having been in the IRP, and that this is therefore, for whatever reason, possibly a prerequisite for achieving this advanced age (Strindhall *et al.*, 2007). Thus, this cluster of immune parameters, the IRP, including seropositivity for CMV, and characterized by accumulations of clonal expansions of late-differentiated CD8⁺ T cells, many of which are specific for CMV antigens, may be considered a genuine hallmark feature of immunosenescence because it has actually been demonstrated to correlate with a robust clinical outcome, i.e. mortality.

25.3 Reasons for the observed effects of age on immunity

25.3.1 Haematopoiesis: age effects on stem cells and the origin of the constantly renewing immune system

One potential reason for any age-associated functional decline in immunity would be predicted to be associated with changes in the haematopoietic stem cells (HSC), which must continuously replenish the blood and immune system throughout life, giving rise to all cellular (lymphoid and myeloid) components (see Fig. 25.3, left). Little is known in humans, but in mice age-related changes within the HSC compartment have been observed. These may be dependent (intrinsic) or independent of cellular ageing of HSCs themselves (e.g. stem cell niche changes) (see Fig. 25.4). There are increased numbers of HSC with decreased homing efficiency, and propensity, to generate higher proportions of myeloid cells. HSCs from old animals show more DNA damage and increased levels of intracellular ROS (Dykstra and de Haan, 2008; Warren and Rossi, 2009). Genomic damage and epigenetic instability also increase the likelihood of malignant transformation, resulting in many myeloproliferative disorders markedly more common in the elderly than the young, in humans as well (Fig. 25.4). There are indications that ageing does not change individual HSCs themselves, but rather their clonal composition is different (Cho *et al.*, 2008).

Recent results support a model in which an aged microenvironment facilitates the mono- or oligoclonality and might play a role in the elevated incidence of leukaemia in the old population (Vas *et al.*, 2012). Data from limiting dilution analysis of whole bone marrow (BM) or assays of purified stem cells (Dykstra and de Haan, 2008; Beerman *et al.*, 2010) support this notion, as the HSC pool consists of a heterogeneous mixture of cells, each with different characteristics. A shift in the representation of these HSC subsets with age could be caused by stochastic differences intrinsic to self-renewal paths, or could be a response to some aspect of the microenvironment or stem cell niche associated with ageing processes in other tissues. The bone marrow microenvironment is a complex assembly of cells

including fibroblasts, megakaryocytes, osteoblasts, osteoclasts, adipocytes, chondrocytes and myoblasts, which form the (heterogeneous) stem cell niche. This is progressively degraded and replaced with adipose tissue with advancing age, suggesting an early potential impact of ageing on the generation of cells differentiating into all the components of the immune system (Compston, 2002; Gruver *et al.*, 2007).

25.3.2 Thymic involution: reduction in naïve T-cell output

The age-dependent involution of the thymus (see Fig. 25.4) is associated with subsequent losses in repertoire diversity of T lymphocytes due to decreased production of naïve cells. Loss of thymic function and TCR diversity is thought to contribute to weaker immunosurveillance of the elderly, including increasing incidence of diseases, such as cancers, autoimmunity and opportunistic infections (Lynch *et al.*, 2009). Despite the belief that thymic involution is one of the major contributors to immunosenescence, there is much confusion over the initiation of the process, because it is not induced by senescence as the organ starts involuting from a young age, as early as the first year of life in humans. This must therefore be viewed as a developmental variable markedly influencing events much later in life. The overall size of the thymus apparently remains relatively constant after puberty, but, as with the bone marrow, gross changes occur with age. These are faster in the thymus and include expansion of the perivascular spaces filled with connective tissue and contraction of the structured thymic epithelium where thymopoiesis occurs. These are progressive changes subject to much inter-individual variation, eventually resulting in shrinkage of thymic medullary and cortical tissue to small islands surrounded by adipose and fibrous tissue (Gruver *et al.*, 2007). In parallel, this results in reduction of the relative levels of thymocytes and stromal cells, but some degree of thymopoiesis does continue throughout life, or at least up to quite advanced age. This thymic productivity is, of course, also dependent on the entry of T-cell progenitors generated in the bone marrow, production of which is also compromised with age. They also show poorer proliferative capacity, essential to increase the small numbers of progenitors available. All these changes conspire to lower thymic productivity with age (Hakim and Gress, 2005; Hakim *et al.*, 2005).

Thymopoiesis may also be significantly affected by the endocrine system through such systemic hormones as growth hormone (GH) and insulin-like growth factor (IGF-1). It was shown that thymocytes express receptors for one or more hormones that include GH, IGF-1 and sex steroids (Savino, 2007; Dykstra and de Haan, 2008). Thus, declining levels of GH and IGF-1 may reduce lymphopoiesis, while the post-pubertal increase in androgens, oestrogens and progesterone contributes to the thymic involution, particularly in males. Under certain circumstances, the thymus can also undergo transient or reversible thymic regression, termed acute thymic involution. Transient involution or atrophy is often associated with physiological stress such as infections, malnutrition and pregnancy and is believed to be mediated, in part, by factors of the neuroendocrine system (Shanley *et al.*, 2009).

Altered thymic activity is influenced by environmental conditions (prenatal and during early life), genetics, sexual dimorphism (females and males exhibit a different pattern of thymic involution) and thymic stroma (Gui *et al.*, 2012). Several studies suggest that the thymic stroma has a crucial role to play in driving thymic involution. Therefore, the central factor regulating thymic involution seems to be within the thymic stroma itself. Indeed, several investigations suggest that the thymic epithelial cell (TEC) is a decisive component. Age-associated decreased production by TEC of factors like the cytokine IL-7, which is necessary for thymocyte maturation, and of fibroblast growth factor (FGF)-7 production by thymocytes, also plays an important role (Alpdogan *et al.*, 2006). Thus, factors including systemic hormonal shifts and intrinsic cytokine programmes within thymocytes and TEC cells can all affect thymopoiesis.

25.3.3 Alterations in antigen presenting cells leading to altered T-cell activation

As discussed above, quantitative and qualitative changes of T-lymphocyte status, both in terms of distribution of differentiation stages and production of naïve T cells, are implicated in the age-related remodelling of the immune system (see Fig. 25.4). To recap, fewer naïve T cells can be produced in later life due to bone marrow and thymic degeneration. Pathogen exposure throughout life results in progressive conversion of naïve cells to memory cells, with chronic pathogen exposure likely to eventually result in immune exhaustion and loss of immunosurveillance capacity.

At the cellular level, in order for T cells to be activated against pathogens, antigens derived from these pathogens must first be taken up by professional APCs, usually DCs, processed and presented to T cells. An age-associated dysbalance or poor function of APCs could clearly represent a first hurdle for triggering adaptive immunity. It was suggested early on that a major defect in old individuals lay at the level of the APC not the T cell (Effros and Walford, 1984).

Nonetheless, several later studies indicated that APCs seem relatively intact in the elderly, but there are clearly certain quantitative and qualitative differences between old and young (Lung *et al.*, 2000). For example, chemotaxis and phagocytosis have been reported to be impaired in DCs from the elderly (Agrawal *et al.*, 2007a). The same investigators reported that naïve CD8⁺ T cells are stimulated by DCs from young and elderly people equally well, but naïve CD4⁺ T cells are not so well activated by old DCs (Agrawal *et al.*, 2007a,b, 2008). Altered proportions of different types of APC may be partly responsible for these findings (Della Bella *et al.*, 2007); this could also explain the altered cytokine production pattern in DCs from the elderly, which may be biased towards greater IL-10, a suppressive cytokine, but less IL-12, the main cytokine involved in activation of T cells (Uyemura *et al.*, 2002). Clearly, such changes would affect the differentiation and function of naïve T cells stimulated by such DCs. Such effects may be of great clinical significance in elderly people, as illustrated by the findings of van Duin *et al.* (2007) that prevaccine determination of the expression of co-stimulatory B7-

family molecules in activated monocytes predicted influenza vaccine responses in young and older adults. Here, the elderly were deficient in the upregulation of costimulatory molecules on the surface of APCs with clear clinical consequences (van Duin *et al.*, 2007). This altered cytokine production by DCs and changes in signalling pathways are likely to account for at least some of the differences observed (Agrawal *et al.*, 2007a). Reduced phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3 in the elderly mice results in higher activation threshold for gene transcription and feedback upregulation of co-stimulatory molecules (Wong *et al.*, 2010).

In a preclinical model, age-associated functional impairment of DCs led to decreased T cell-mediated anti-tumour immunity, suggesting that not only influenza vaccination but cancer immunosurveillance could well be detrimentally affected by age-associated changes to APCs (Grolleau-Julius *et al.*, 2008). There is also increasing evidence of an involvement of aged DC in autoimmunity and age-associated chronic diseases (Agrawal *et al.*, 2012).

25.4 Conclusion

Human ageing is associated with a decline in the capacity to respond to vaccines, with increases in the frequency and severity of infectious diseases, and with increased incidence of chronic inflammatory and autoimmune disorders. Some of these changes are highly likely to be influenced by nutritional parameters differing between young and older people. This is an area which has been little explored, but where interest in nutritional–microbiomic–immune interactions overlaying the purely nutritional aspects of immune function interposed on the genetic background, and the effect of age and environmental exposures thereon is becoming of paramount importance in understanding and manipulating human immune function. Dissecting the different influences and interactions of these multitudinous factors will be an immense challenge. As we have seen above, while innate and adaptive immunity are different in the elderly, the clinical impact of these changes is not clear, and mechanisms of, and markers for, immunosenescence are controversial. It is also difficult to distinguish age effects from age-related diseases. Nevertheless, it is clearly emerging that immunological markers can be potent indicators of general health status. Comparing inflammatory (non-clinical) and metabolic (clinical) markers parameters has been suggested to predict mortality in old populations better than clinical factors alone (Goldman *et al.*, 2006; Pawelec and Larbi, 2008). Greater sophistication in assembling clusters of parameters, including, but not limited to, immunological markers is likely to be more informative in future.

Currently, the decreased responsiveness of the elderly to vaccination represents a major public health issue. Strategies are required to select individuals with better or poorer immune response capacity to address and compensate for the effects of immunosenescence. These could include improving diet and exercise to address the effects of frailty, which also has beneficial ‘knock-on’ effects on immune

function (Grubeck-Loebenstein *et al.*, 2009). Countering the effects of immunosenescence might also be approached by altering vaccine formulations and using better adjuvants tailor-made for the elderly, and taking into account the negative effects of CMV infection (Derhovanessian *et al.*, 2013). Giving immunostimulatory agents and anti-inflammatory cytokines might also be effective. Increasing DC activity, and enhancing B and T lymphocytes using factors like IL-2, IL-10, keratinocyte growth factor, thymic stromal lymphopoietin, as well as leptin, may have beneficial effects. More effective vaccines have been successfully tested in animals, for example using high antigen doses, DNA vaccines with immunostimulation, virosomal vaccines and vaccines containing new adjuvants. These approaches could possibly be adapted and translated into human therapy in the near future (Ongradi and Kovessi, 2010).

25.5 Future trends

To facilitate real progress in understanding and modulating human immunosenescence, future work will need to focus more on areas such as furthering understanding of the basis of reduced vaccination responses in older adults, who have a profoundly compromised response to vaccination. The basis for this is, however, poorly understood. We need to define the clinically-relevant components of immunosenescence that are important for maintaining immune health in old age. Beyond the obvious consequences for increased risk of infection, dysregulated immunity has a broad influence on health and affects many organ systems.

Thus, research will establish immune signatures associated with decreased efficacy of preventative strategies, not only vaccination, but also age-associated increased risks of general infections (bacterial, fungal, viral and parasitic), chronic inflammatory diseases, dementia, autoimmune diseases, neoplasia and cardiovascular disease. Such data will allow prediction and intervention to ameliorate these widespread and clinically significant conditions. For this, it will be necessary to determine the factors contributing to immunosenescence. It is now clear that a variety of genetic and environmental factors impact upon health in old age, including effects on immunity. However, the relative contribution of these factors to immunosenescence will have to be more accurately established. These variables clearly include nutrition (micro and macro) and obesity, as well as gender and ethnicity, genetic background, psychosocial parameters (including stress), mental well-being, socio-economic status, early life events and exposures, physical activity, parity/multiparity and different chronic infections. It will be necessary to build up a solid evidence base in order to develop effective personalized interventions, both lifestyle and pharmacologic, to extend healthspan. It will be essential to integrate these assessments of immune signatures into more ongoing and planned longitudinal studies. Because dysregulated immunity exerts systemic effects on many organ systems, and contributes to pathology, it is vital to include the assessment of immune parameters in most studies.

However, although the emphasis will remain on human research, it will also be crucial to develop and validate improved *in vitro* and animal models to better understand human immunosenescence. The *in vitro* approaches could, for example, include artificial immune organs, improved physiological culture conditions, automation and high-throughput approaches for drug screening. Current animal models are predominantly in rodents, the immune systems of which may not accurately reflect the human, so we would foresee better models including non-human primates, mini-pigs, etc., as well as the use of *in vivo* imaging of immune processes in other appropriate models (for example, green fluorescent protein, GFP-expressing immune cells in fish and amphibians).

We should see more awareness of the requirement for immune monitoring in the assessment of existing interventions in humans. Interventions such as improved vaccination regimens, prophylactic vaccinations (e.g. for CMV), nutritional interventions (micro- and macronutrients), use of existing drugs (e.g. anti-inflammatory drugs, statins, therapeutic antibodies, antidepressants) and physical activity should be rapidly implementable and could be proven effective in the near future. Finally, we will also see the development of more cost-effective and practical diagnostic and monitoring tools for immunosenescence – with a view to facilitating inclusion of the measurement of immunological parameters in longitudinal studies as well as validation of interventions.

25.6 Sources of further information and advice

Professional bodies including interest groups focussed on immunosenescence are a rarity. Within learned societies, working groups concentrating on age and immunity are beginning to emerge (e.g. the Affinity Group on Lymphocyte Immunosenescence & Differentiation of the British Society for Immunology, see <http://www.immunology.org/page.aspx?pid=1184>). Learned societies with an interest in the biology of ageing may also include topics related to immunosenescence in their meetings and activities, eg. the German Association for Aging Research (<http://www.alternsforschung.org/>), the Gerontological Society of America (<http://www.geron.org/>), or the American Aging Association (<http://www.americanaging.org/>). There are no textbooks on immunity and ageing, but some 'Handbooks' have been recently published on, or including, this topic (e.g. *Handbook of Models of Human Aging*, Ed. P.M. Cohn, Elsevier, ISBN-13: 978-0-12-369391-4, 2006; *Immunosenescence*, Ed. G. Pawelec, Springer, ISBN: 978-0-387-76840-3, 2007; *Handbook on Immunosenescence*, Eds T. Fülöp, C. Franceschi, K. Hirokawa and G. Pawelec, Springer, ISBN: 978-1-4020-9062-2, 2009). There is one specialist open-access journal dedicated to publishing high-quality peer-reviewed papers specifically relevant to immunity and aging (see <http://www.immunityageing.com/>).

25.7 References

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26

Exercise, nutrition and immunity

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Abstract: The chapter begins by discussing the impact of intensive exercise training on immune function and susceptibility to infection in athletes. It then reviews the evidence that diet and nutritional supplements can modify immune responses to exercise and reduce infection incidence, symptom severity or duration in endurance athletes.

Key words: exercise training, endurance athletes, susceptibility to infection, diet and nutritional supplements, innate and acquired immunity.

26.1 Introduction

The immune system is involved in tissue repair after injury and in the protection of the body against potentially damaging (pathogenic) micro-organisms such as bacteria, viruses, and fungi. Several forms of stress, both physical and psychological, including a heavy schedule of training and competition, can lead to immunodepression, placing athletes at a greater risk for opportunistic infections, particularly upper respiratory tract infections (URTIs). These are the most common infections in athletes and the majority of URTIs are caused by a virus rather than a bacteria (and thus do not benefit from antibiotic therapy). In themselves, these episodes of URTI are generally trivial, but they can interrupt training or cause an athlete to miss (or under-perform in) important competitions.

Many factors influence exercise-induced immunodepression (e.g. physical, environmental and psychological stresses), but nutrition is a factor that plays a critical role. This chapter examines the role of nutrition in exercise-induced immunodepression and in particular the nutritional practices that could worsen, prevent or alleviate depressed immunity associated with heavy physical exertion. Particular attention is paid to the evidence that so-called ‘dietary immunostimulants’

can modify immune responses to exercise and/or reduce the risk of infection for athletes involved in heavy training.

26.2 Effects of exercise on the immune system

Athletes engaged in heavy training programmes, particularly endurance events, appear to be more susceptible to infection than the general population. For example, sore throats and common cold or flu-like symptoms are more common in athletes. Some convincing evidence suggests that this increased susceptibility to infection arises because of a depression of immune system function (Peters, 1997; Pedersen *et al.*, 1998; Nieman, 2000; Gleeson, 2005, 2007).

The circulating numbers and functional capacities of leukocytes may be decreased by repeated bouts of intense, prolonged exercise (Nieman, 1997; Gleeson, 2005, 2006a, 2007). The cause may be increased levels of stress hormones (e.g. adrenaline and cortisol) and anti-inflammatory cytokines (e.g. interleukin (IL)-6, IL-10 and IL-1 receptor antagonist) during exercise and entry into the circulation of less mature leukocytes from the bone marrow (Gleeson, 2005). Drops in the blood concentration of glutamine have also been suggested as a possible cause of the immunodepression associated with heavy training, although the evidence for this is less compelling (Hiscock and Pedersen, 2002).

During exercise, exposure to airborne bacteria and viruses increases because of the higher rate and depth of breathing. An increase in gut permeability may also allow entry of gut bacterial endotoxins into the circulation, particularly during prolonged exercise in the heat. In contact sports, skin abrasions may occur increasing the risk of transdermal infections. In some sports the competitors may be in close proximity to large crowds. Air travel to foreign countries may be involved. Hence, the cause of the increased incidence of infection in athletes is most likely multifactorial (Gleeson 2005, 2006). A variety of stressors (physical, psychological, environmental and nutritional) suppress immune function, and these effects, together with increased exposure to potentially disease-causing pathogens, make the athlete more susceptible to infection (see Fig. 26.1).

The relationship between exercise and susceptibility to infection has been modelled in the form of a J-shaped curve (Nieman, 1994) as illustrated in Fig. 26.2. This model suggests that, whereas engaging in moderate activity may enhance immune function above levels seen in sedentary individuals, excessive amounts of prolonged, high-intensity exercise induce detrimental effects on immune function. Although the literature provides strong evidence in support of the latter point, relatively little evidence is available to suggest any clinically significant difference in immune function between sedentary and moderately active persons. Matthews *et al.* (2002) reported that the regular performance of about two hours of moderate exercise a day is associated with a 29% reduction in risk of URTI compared with a sedentary lifestyle. This finding emphasizes that the benefit of regular, moderate exercise in improving resistance to infection is rather small.

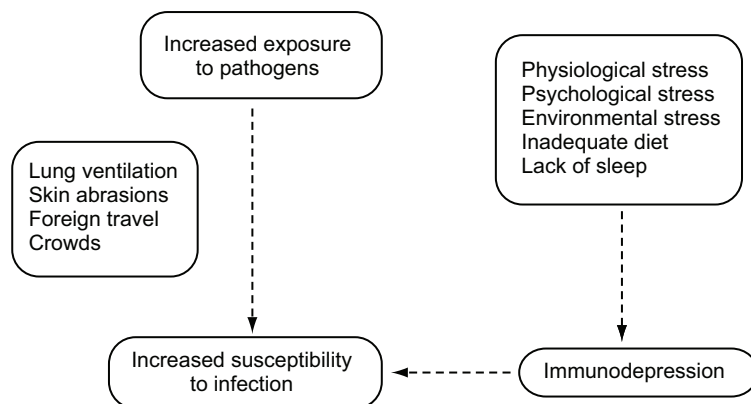


Fig. 26.1 Factors affecting infection risk in athletes. This figure was published in *Immune Function in Sport and Exercise*, Gleeson, M. (editor), page 254, Copyright Elsevier (2005).

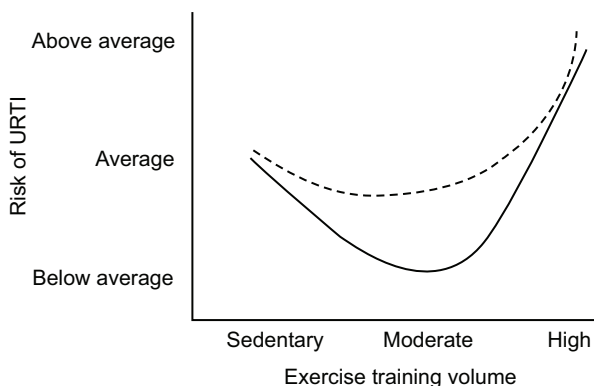


Fig. 26.2 The original 'J-shaped model' of the relationship between infection risk and exercise training load (solid line from Nieman, 1994). The dashed line may give a more realistic interpretation of the relationship. This figure was published in *Immune Function in Sport and Exercise*, Gleeson, M. (editor), page 116, Copyright Elsevier (2005).

26.2.1 Acute effects of exercise on immune function

An acute bout of physical activity is accompanied by responses that are similar in many respects to those induced by infection (Nieman, 1997; Gleeson, 2005, 2007). A substantial increase in the number of circulating leukocytes (mainly lymphocytes and neutrophils) occurs, the magnitude of which is related to both the intensity and duration of exercise. Increases also occur in the plasma concentrations of various substances that influence leukocyte functions, including acute-phase proteins such as C-reactive protein, activated complement fragments and inflammatory and anti-inflammatory cytokines such as interferon (IFN)- γ , tumour necrosis factor- α , IL-1, IL-2 and IL-6. Other changes also occur in response to exercise, including

risers in the plasma concentration of several hormones (e.g. adrenaline, cortisol, growth hormone and prolactin) that have immunomodulatory effects.

Natural killer cell activity, various measures of T and B cell function, upper airway neutrophil function, salivary immunoglobulin A (IgA) concentration, granulocyte oxidative burst activity, skin delayed-type hypersensitivity response and major histocompatibility complex (MHC) II and Toll-like receptor expression in macrophages are suppressed for at least several hours during recovery from prolonged, intense endurance exercise (Nieman, 1997; Gleeson, 2005, 2007). These immune changes occur in several compartments of the immune system and body (e.g. the skin, upper respiratory tract mucosal tissue, lungs, blood, muscle and peritoneal cavity). These changes during early recovery from exercise appear to weaken the potential immune response to pathogens and possibly provide an 'open window' for infection (Pedersen and Bruunsgard, 1995). During this 'open window' of impaired immunity (which may last between three and 72 hours, depending on the immune measure), viruses and bacteria may gain a foothold, increasing the risk of subclinical and clinical infection. Several epidemiological studies have shown that athletes engaging in marathon and ultramarathon race events are at increased risk of URTIs, particularly in the one to two weeks following such events. For example, both Peters and Bateman (1983) and Nieman *et al.* (1990) described a substantially higher (two-fold to six-fold) frequency of self-reported symptoms of URTI in athletes who completed long-distance foot races compared with control runners who did not compete in the events. In recent years it has been questioned whether or not the illness symptoms reported by athletes are all due to infections and it has been suggested that a substantial proportion of so-called URTIs may rather reflect non-infectious inflammation of the airways caused by allergic responses or oral breathing of cold dry air and/or airborne pollutants (Spence *et al.*, 2007). However, in general, these epidemiological and exercise immunology studies support the viewpoint that heavy exercise workloads increase URTI risk through altered immune function.

26.2.2 Chronic effects of exercise training on immune function

Circulating numbers of leukocytes are generally lower in athletes at rest compared with sedentary people, and neutrophil function may be slightly depressed; however, acquired immunity is generally unaffected by athletic endeavour (Gleeson, 2005). Thus, in general, the resting immune status of athletes differs little from that of non-athletes and it is the transient acute changes in immune function that take place during and after prolonged workouts that provide a more likely explanation of the increased risk of URTIs (Nieman, 2008). However, when exercise training loads are particularly high, several studies have reported the development of a chronic state of moderate immune depression (Gleeson, 2005). For example, levels of secretory immunoglobulins, such as salivary IgA, are lower during periods of intensified training, as are T-lymphocyte CD4⁺/CD8⁺ ratios and *in vitro* antigen-stimulated lymphocyte proliferation and cytokine production. Neutrophil

functions are also reported to be further depressed after periods of intensified training (Gleeson 2005, 2006a). Thus, with chronic periods of heavy training, both innate and adaptive immunity are depressed and several studies report a high incidence of URTIs during periods of intensive training and competition in athletes (Gleeson *et al.*, 1999; Fahlman and Engels, 2005; Clancy *et al.*, 2006; Spence *et al.*, 2007; Nieman *et al.*, 2007, 2008; Neville *et al.*, 2008). A retrospective study of 852 German athletes over a 12-month period showed that the risk of URTI was highest in endurance athletes who experienced significant stress and poor sleep quality (Konig *et al.*, 2000). Thus, infection risk is likely to be highest in athletes who engage in repeated cycles of heavy, prolonged exertion and are also subject to other stressors including mental anxiety, lack of sleep, weight loss, inadequate nutrition and frequent exposure to novel pathogens. Within both the general and the athletic population there is a range of immunocompetency due to genetic differences, age, gender and lifestyle habits, and it is likely that some individuals will be more prone to succumb to illness episodes when exposed to the stressors associated with the training needs of the elite athlete.

26.3 Nutritional practices of athletes influencing immune functions

Adequate nutrition and, in particular, appropriate intakes of energy, protein, vitamins and minerals are essential to maintain the body's natural defences against disease-causing micro-organisms (pathogens). Thus, athletes are generally advised to consume a sound diet that meets their energy needs and contains a variety of foods (Jeukendrup and Gleeson, 2010). It is important to remember that any sustained deficiency of an essential vitamin or mineral will result in ill health (Calder *et al.*, 2002), and it is extremely unlikely that an unhealthy athlete will perform to the best of his or her potential. Therefore, the key to maintaining an effective immune system is to avoid deficiencies of the nutrients that play an essential role in immune cell functions.

Inadequate protein–energy intake or deficiencies of certain micronutrients (e.g. iron, zinc and vitamins B6 and B12) decrease immune defences against invading pathogens and make the individual more susceptible to infection (Scrimshaw and SanGiovanni, 1997; Calder *et al.*, 2002). It is well accepted that an inadequate intake of protein impairs host immunity with particularly detrimental effects on the T-cell system, resulting in increased incidence of opportunistic infections (Chandra, 1997). Essentially all forms of immunity have been shown to be affected by protein malnutrition in humans, depending on the severity of the protein deficiency relative to energy intake. While it is unlikely that athletes would ever reach a state of such extreme malnutrition unless dieting very severely or suffering from an eating disorder, some impairment of host defence mechanisms is observed even in moderate protein deficiency. In a study on judoists experiencing combined exercise and weight loss, significant falls in serum immunoglobulins and complement proteins were observed after only four weeks of dietary restriction that resulted in

an average body mass loss of 2.8 kg (Umeda *et al.*, 2004). In a similar study by the same researchers, falls in neutrophil function were also observed after a 20-day period of combined exercise and dietary restriction (Yaegaki *et al.*, 2007). Thus, care should be taken to ensure adequate protein (and micronutrient) intakes during periods of intentional weight loss and it should be recognized that athletes undergoing weight reduction are more prone to infection. The practice of Muslim athletes to abstain from food and fluid intake from dawn to sunset during the month of Ramadan appears to have limited impact on markers of immune system status (Chaouachi *et al.*, 2009), although it may impact on their ability to recover quickly from intensive training sessions and to perform at peak level in competition (Burke, 2010).

Several vitamins are essential for normal immune function. Deficiencies of fat-soluble vitamins A, D and E and water-soluble vitamins folic acid, B6, B12 and C impair immune function and decrease the body's resistance to infection (Calder *et al.*, 2002). Correcting existing deficiencies with specific vitamin supplements can be effective in restoring immune function to normal levels. Several minerals are known to exert modulatory effects on immune function, including zinc, iron, magnesium, manganese, selenium and copper yet, with the exception of zinc and iron, isolated deficiencies are rare (Calder *et al.*, 2002). Field studies consistently associate iron deficiency with increased morbidity from infectious disease. Furthermore, exercise has a pronounced effect on both zinc and iron metabolism. Requirements for these minerals are certainly higher in athletes compared with sedentary people because of increased losses in sweat and urine (Jeukendrup and Gleeson, 2010). However, it is also recognized that excesses of iron and zinc can actually impair immune function. Hence, supplements should be taken only as required and regular monitoring of an athlete's iron status (serum ferritin and blood haemoglobin) and zinc status (erythrocyte zinc) is probably a good idea.

Dietary surveys show that most athletes are well able to meet the recommended intakes for vitamins and minerals by eating everyday foods. Those at risk of suboptimal intakes of these micronutrients include athletes who restrict their energy intake, especially over long periods, usually in an attempt to lose weight (fat) and athletes who follow eating patterns with restricted food variety and reliance on foods with a poor micronutrient density (Jeukendrup and Gleeson, 2010). Energy-restricted diets are not uncommon in sports where leanness or low body mass confers a performance advantage (e.g. gymnastics, figure skating, and endurance running) or is required to meet certain bodyweight criteria (e.g. boxing, martial arts, weightlifting and rowing). Some athletes adopt diets that are very high in carbohydrate content at the expense of protein and fat. By avoiding foods high in animal fat, athletes are also reducing their intake of fat-soluble vitamins and essential fatty acids. Many athletes avoid dairy produce to minimize intake of saturated fat but, consequently, they are omitting from their diet major sources of vitamin D, B-group vitamins and calcium, all of which play roles of varying importance in maintaining immune function. If fat intake is a concern, then athletes should select non-fat or low-fat dairy products which provide the same (or higher)

levels of calcium, vitamin D and vitamin B12 as full-fat dairy products. Only recently has the importance of vitamin D for immune function been recognized and it is apparent that vitamin D deficiencies in athletes are not uncommon (Willis *et al.*, 2008).

In general, a broad-range multivitamin/mineral supplement is the best choice to support a restricted food intake, and this may also be suitable for the travelling athlete in situations where food choices and quality may be limited. It is also worth remembering that certain infections can also adversely affect nutritional status by causing appetite suppression, malabsorption, increased losses of endogenous nutrients and increased nutrient requirements (Calder *et al.*, 2002).

26.4 Nutrition strategies to limit exercise-induced immune depression

Certain supplements may improve immune function and reduce infection risk in immunocompromised individuals (Nieman, 2008; Jeukendrup and Gleeson, 2010). This strategy has proved effective for some immunocompromised groups including the frail elderly and patients recovering from burns, trauma and surgery. While there are many nutritional supplements including arginine, glutamine, bovine colostrum, whey protein, vitamin C, probiotics, zinc and herbals such as echinacea on the market that are claimed to enhance or 'boost' immunity, such claims are often based on selective evidence of efficacy in animals, *in vitro* experiments, children, the elderly or clinical patients in severe catabolic states, and direct evidence for their efficacy for preventing exercise-induced immune depression or improving immune system status in athletes is usually lacking. Some scientists question the value of using immunonutrition support for athletes because blunting the immune, inflammatory and free radical perturbations that occur during exercise could interfere with the important signalling mechanisms that promote training adaptations (Ristow *et al.*, 2009).

The best evidence of how to limit the immunodepressive effects of heavy exertion supports the implementation of appropriate rest periods within the training micro-cycle and the use of a high carbohydrate diet and carbohydrate ingestion (about 30–60 g per hour) during prolonged workouts, which lowers circulating adrenaline and cortisol levels and delays the appearance of symptoms of overreaching during intensive training periods (Gleeson *et al.*, 2004; Halson *et al.*, 2004). Several placebo-controlled studies in runners and cyclists have shown that carbohydrate ingestion (usually in the form of a beverage) during prolonged exercise is effective in attenuating detrimental changes in some aspects of immune function (Gleeson, 2006b; Nieman, 2008). However, evidence is currently lacking to demonstrate that this translates to a reduced incidence of URTIs following competitive events.

The consumption of beverages during exercise not only helps prevent dehydration (which is associated with an increased stress hormone response) but also helps to maintain saliva flow rate during exercise. Saliva contains several proteins with

antimicrobial properties including immunoglobulin A, lysozyme and α -amylase. Saliva secretion usually falls during exercise, but regular fluid intake during exercise can prevent this (Bishop *et al.*, 2000).

Relatively little is known about the potential contribution of dietary fatty acids to the regulation of exercise-induced modification of immune function. Two groups of polyunsaturated fatty acids (PUFA) are essential to the body: the ω -6 (n-6) series, derived from α -linoleic acid and the ω -3 (n-3) series, derived from α -linolenic acid. These PUFA cannot be synthesized in the body and therefore must be derived from the diet. There are reports that diets rich in either of these PUFA improve the conditions of patients suffering from diseases characterized by an over-active immune system, such as rheumatoid arthritis; that is to say they have anti-inflammatory effects (Calder *et al.*, 2002). One study in untrained men reported that the inflammatory response to eccentric exercise-induced muscle damage, evaluated by increases in circulating IL-6 and C-reactive protein, was attenuated by a supplement containing a mixture of docosahexaenoic acid (DHA) (a n-3 PUFA), tocopherols and flavonoids (Phillips *et al.*, 2003). However, it has been suggested that high intakes of PUFA may exert an undesirable influence on inflammation and immune function during and after exercise (Konig *et al.*, 1997) and some human studies have shown that dietary supplements with n-3 PUFA (e.g. eicosapentaenoic acid, EPA) decrease natural killer cell activity in healthy humans (Thies *et al.*, 2001). A recent study, however, indicated that supplementation with a combination of n-3 PUFA (EPA and DHA acid) for six weeks had no effect on selected immune responses to prolonged exercise, although in this study natural killer cell activity was not measured (Nieman *et al.*, 2009a). A follow-up study by the same group has generated some concern after reporting an increase in the oxidative stress marker, F_2 -isoprostane in plasma after exercise when trained cyclists were supplemented daily with the same n-3 PUFA combination (McAnulty *et al.*, 2010). More research is needed on the effects of altering essential fatty acid intake on immune function after exercise and during periods of heavy training.

Although it is not known whether hard training increases the need for dietary antioxidants – as the body naturally develops an effective defence with a balanced diet and endogenous antioxidant defences actually improve with exercise training – some recent evidence suggests that regular intake of relatively high doses of antioxidant vitamins can also reduce the cortisol response to prolonged exercise (Fischer *et al.*, 2004; Davison and Gleeson, 2006). Other supplements for which there is some evidence of benefit in reducing infection incidence and/or improving immune function in athletes include probiotics, quercetin and bovine colostrum. The following sections review the evidence for the various ‘immune-enhancing’ nutrients and supplements that have been tested in athletes. Their proposed mechanisms of action and evidence of efficacy are summarized in Table 26.1.

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Table 26.1 Nutrition supplements that are claimed to boost immunity in athletes: proposed mechanisms of action and summary of evidence for efficacy

Nutrition supplement and evidence rating	Proposed mechanism of action	Evidence of efficacy
n-3 PUFAs ○○○○○	Exert anti-inflammatory effects post-exercise	Some evidence of limiting inflammation after muscle damaging eccentric exercise in humans, but no evidence of reducing infection risk in athletes
Vitamin E ●○○○○	An essential fat-soluble antioxidant vitamin that quenches exercise-induced reactive oxygen species (ROS) and augments immunity. Good evidence for some immune enhancing effects in the frail elderly	No evidence of similar benefit for younger healthy humans or athletes
Vitamin C ●●○○○	An essential water-soluble antioxidant vitamin that quenches ROS and augments immunity. Reduces interleukin-6 and cortisol responses to exercise in humans	Relatively small effects on cortisol compared with carbohydrate; immune measures no different from placebo. Some evidence of efficacy in reducing respiratory infection incidence after ultramarathon events
Glutamine ●○○○○	Non-essential amino acid that is a precursor in the synthesis of nucleic acids and important for rapidly dividing cells. Also an important fuel for immune cells. Plasma concentration of glutamine falls during prolonged exercise	Supplementation before and after exercise does not alter immune perturbations despite maintenance of plasma glutamine
Carbohydrate ●●●○○	Maintains blood glucose during exercise, lowers stress hormones and thus counters immune dysfunction	Ingestion of carbohydrate (30–60 g/h) attenuates stress hormone and some (but not all) immune perturbations during exercise but, only very limited evidence that this modifies infection risk in human athletes
β-glucans ●●○○○	Polysaccharides derived from the cell walls of yeast, fungi, algae and oats that stimulate innate immunity	Effective in mice inoculated with influenza virus, but human study with athletes showed no benefits

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Echinacea ●○○○○	Herbal extract that is a popular supplement among athletes. Claimed to enhance immunity via stimulatory effects on macrophages and there is some <i>in vitro</i> evidence for this	Early human studies indicated possible beneficial effects but more recent, larger scale and better controlled studies indicate no effect of Echinacea on infection incidence or cold symptom severity
Probiotics ●●●○○	Probiotics are live micro-organisms which when administered orally for several weeks, can increase the numbers of beneficial bacteria in the gut. This has been associated with a range of potential benefits to gut health, as well as modulation of immune function	Human studies show improvements in some aspects of acquired immunity and reduced incidence of respiratory illness and gastrointestinal problems
Quercetin ●●●○○	A plant flavonoid; <i>in vitro</i> studies show strong anti-inflammatory, antioxidant, and antipathogenic effects. Animal data indicate increase in mitochondrial biogenesis and endurance performance	Human studies show some reduction in illness rates during short periods of intensified training and mild stimulation of mitochondrial biogenesis and endurance performance in untrained subjects
Quercetin with epigallocatechin 3-gallate ●●●○○	Flavonoid mixture that has anti-inflammatory and antioxidant effects and immune function improvement, above that of quercetin alone	Human study showed strong anti-inflammatory effect, with modest antioxidant effect and improvement in innate immunity
Bovine colostrum ●○○○○	First milk of the cow that contains antibodies, growth factors and cytokines. Claimed to improve mucosal immunity and increase resistance to infection	One study suggests an effect in elevating salivary IgA in human endurance runners, but no evidence yet that this modifies infection risk
Zinc ●○○○○	Zinc deficiency results in impaired immunity, and zinc deficiency is not uncommon in athletes. An essential mineral that is claimed to reduce incidence and duration of colds	No evidence for reduced infection incidence with zinc supplementation in humans. Some (but not all) human studies suggest a reduction in duration of cold symptoms if zinc gluconate lozenges are administered within 24 h of cold symptom onset. Unlikely to be of any real benefit to athletes unless they are zinc deficient

The scientific evidence is indicated with ●●●●● meaning very strong evidence and ○○○○○ meaning limited to no evidence.

26.5 The evidence for nutritional countermeasures to exercise-induced immunodepression

26.5.1 Carbohydrate

The importance of adequate carbohydrate availability for maintenance of heavy training schedules and successful athletic performance has long been recognized. During periods of heavy training, athletes should consume sufficient carbohydrate to cover about 60% of their energy costs. The recommended daily intake is 8–10 g/kg body mass (b.m.) of carbohydrate for athletes who train for more than two hours/day (Jeukendrup and Gleeson, 2010). These recommendations are principally aimed at restoring muscle and liver glycogen stores to ensure sufficient carbohydrate availability for skeletal muscle contraction for training on successive days and to avoid the development of hypoglycaemia during long training sessions without carbohydrate ingestion.

Since many aspects of exercise-induced immune function impairment seem to be caused by elevated levels of stress hormones, nutritional strategies that effectively reduce the stress hormone response to exercise would be expected to limit the degree of exercise-induced immune dysfunction. The size of the glycogen stores in muscle and liver at the onset of exercise influence the hormonal and immune response to exercise. The amount of glycogen stored in the body is rather limited (usually less than 500 g) and is affected by recent physical activity and dietary carbohydrate intake. When individuals perform prolonged exercise following several days on very low-carbohydrate diets (typically <10% of dietary energy intake from carbohydrate), the magnitude of the stress hormone (e.g. adrenaline and cortisol) and cytokine (e.g. IL-6, IL-10 and IL-1ra) response is markedly higher than on normal or high-carbohydrate diets (Gleeson *et al.*, 1998; Mitchell *et al.*, 1998).

Consumption of carbohydrate during prolonged exercise also attenuates rises in plasma adrenaline, cortisol and cytokines (Nehlsen-Cannarella *et al.*, 1997); attenuates the trafficking of most leukocyte and lymphocyte subsets; prevents the exercise-induced fall in neutrophil function; and reduces the extent of the diminution of mitogen-stimulated T-lymphocyte proliferation (on a per cell basis) after prolonged exercise (Henson *et al.*, 1998). Consuming 30–60 g of carbohydrate per hour during 2.5 hours of strenuous cycling prevented both the decrease in the number and percentage of IFN- γ -positive T lymphocytes and the suppression of IFN- γ production from stimulated T lymphocytes observed with a placebo treatment (Lancaster *et al.*, 2005; Fig. 26.3). IFN- γ production is critical to antiviral defence and it has been suggested that the suppression of IFN- γ production may be an important mechanism leading to an increased risk of infection after prolonged exercise bouts (Northoff *et al.*, 1998). Carbohydrate ingestion during prolonged exercise has also been shown to attenuate the fall in T lymphocyte trafficking into infected airway epithelial tissue that otherwise occurs (Bishop *et al.*, 2009).

Although carbohydrate feeding during exercise appears to be effective in minimizing some of the immune perturbations associated with prolonged strenuous exercise, it does not prevent the falls in the natural killer cell function or

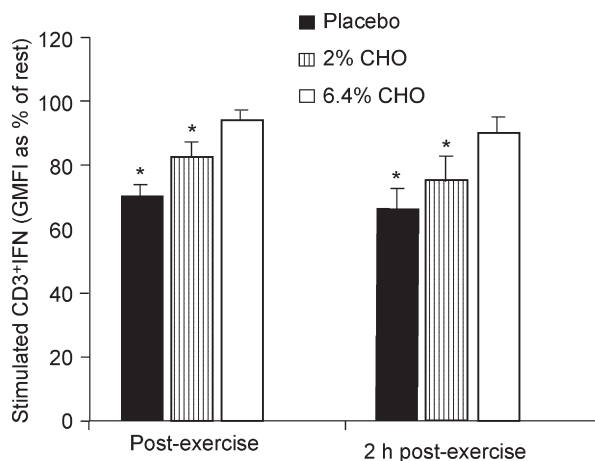


Fig. 26.3 Ingestion of a 6.4%(w/v) carbohydrate beverage (supplying ~40 g carbohydrate per hour) during 2.5 hours of strenuous cycling prevents the suppression of interferon (IFN- γ) production by stimulated T-lymphocytes observed on the placebo control trial. *Significantly lower than pre-exercise, $P < 0.05$. Note that ingesting a smaller amount of carbohydrate (~12 g per hour) as a 2%(w/v) carbohydrate beverage is not as effective. The volume of drinks consumed was 500 ml immediately pre-exercise and 200 ml every 20 minutes during exercise. GMFI, geometric mean fluorescence intensity. Data from Lancaster *et al.* (2005). Reprinted, with permission, from A. Jeukendrup and M. Gleeson, 2010, *Sport nutrition: An introduction to energy production and performance*, 2nd edn (Champaign, IL: Human Kinetics, page 378).

salivary IgA secretion and seems less effective for less demanding exercise such as football (Bishop *et al.*, 1999) or rowing (Nieman *et al.*, 1999) training. Carbohydrate feeding is not as effective in reducing immune-cell trafficking and functional depression when exercise is performed to the point of fatigue (Bishop *et al.*, 2001). Thus, carbohydrate is seen as only a partial countermeasure against exercise-induced immune impairment, and evidence that the beneficial effect of feeding carbohydrate on immune responses to exercise actually translates into a reduced incidence of URTI after prolonged exercise such as marathon races is currently lacking. Although a trend for a beneficial effect of carbohydrate ingestion on post-race URTI was reported in a study of 98 marathon runners (Nieman *et al.*, 2002a), this did not achieve statistical significance and larger scale studies are needed to investigate this possibility.

26.5.2 Glutamine

Glutamine is the most abundant free amino acid in human muscle and plasma and is utilized at very high rates by leukocytes to provide energy and optimal conditions for nucleotide biosynthesis. Indeed, glutamine is important, if not essential to lymphocytes and other rapidly dividing cells, including the gut mucosa and bone marrow stem cells. Glutamine is also required for optimal macrophage phagocytic

activity. Prolonged exercise is associated with a fall in the plasma concentration of glutamine, and such a decrease has been hypothesized to impair immune function. The over-training syndrome is associated with a chronic reduction in plasma glutamine levels, which may be partly responsible for the immunodepression apparent in this condition. Interestingly, evidence indicates that an additional intake of 20–30 g/day of protein can restore depressed plasma glutamine levels in over-trained athletes (Kingsbury *et al.*, 1998).

Several scientists have suggested that exogenous provision of glutamine supplements may be beneficial by preventing the impairment of immune function after prolonged exercise. However, the evidence that oral glutamine supplements reduce the incidence of URTI after endurance events is very limited (Castell *et al.*, 1996). Several studies that have investigated the effect of glutamine supplementation during and after exercise on various indices of immune function have failed to find any beneficial effect. A glutamine solution (0.1 g/kg b.m.) given at 0 minutes, 30 minutes, 60 minutes and 90 minutes after a marathon race prevented the fall in the plasma glutamine concentration but did not prevent the fall in mitogen-induced lymphocyte proliferation and lymphokine-activated natural killer cell activity (Rohde *et al.*, 1998). Similarly, maintaining the plasma glutamine concentration by consuming glutamine in drinks taken both during and after two hours of cycling at 60% VO_2max did not affect leukocyte subset trafficking or prevent the exercise-induced fall in neutrophil function (Walsh *et al.*, 2000). Unlike the feeding of carbohydrate during exercise, glutamine supplements do not seem to affect immune function perturbations, and a review article (Hiscock and Pedersen, 2002) concluded that falls in plasma glutamine are not responsible for exercise-induced immunodepression.

Bassit *et al.* (2002) reported that supplementation (6 g/day for 15 days) of branched-chain amino acids (BCAAs) before a triathlon or a 30-km run prevented the approximately 40% decline in mitogen-stimulated lymphocyte proliferation observed in the placebo control group after exercise. BCAAs are precursors for glutamine, and BCAA supplementation prevented the post-exercise fall in plasma glutamine concentration and was also associated with increased IL-2 and IFN production. More research is needed to resolve these conflicting findings of BCAA and glutamine supplementation on immune responses to exercise.

26.5.3 Caffeine

Caffeine is the most widely consumed drug in Europe and America, and it has long been used by athletes because of the convincing evidence that it improves mental and endurance performance. In 2004 caffeine was removed from the list of banned substances by the International Olympic Committee (IOC). Caffeine originates naturally in 63 species of plants as several types of methylated xanthines. Caffeine and caffeine-like substances can be found in a variety of foods and drinks (now including several sports drinks and energy drinks), but the main sources for these substances are coffee beans, tea leaves, cocoa beans and cola nuts. Coffee accounts for 75% of all caffeine consumption. Caffeine is an adenosine receptor antagonist

and several immune cell types including neutrophils and lymphocytes express adenosine receptors. Furthermore, caffeine ingestion results in elevated circulating adrenaline concentration both at rest and during exercise and so could affect immune cell functions indirectly via actions on adrenoreceptors. At present, there is little information of the effects of caffeine on immune function at rest. Addition of pharmacological doses of caffeine to cell culture media has been associated with a dose-dependent suppression of mitogen-stimulated proliferative responses of human lymphocytes (Rosenthal *et al.*, 1992). However, *in vivo* administration of 18 mg/kg/day of caffeine in rats was associated with a significant increase in mitogen-stimulated T-cell proliferation (Kantamala *et al.*, 1990). In the same study, B cell proliferative responses to mitogen were significantly decreased following administration of 6 mg/kg/day of caffeine.

Recent exercise studies have demonstrated that caffeine compared with placebo ingestion one hour before a bout of intensive endurance exercise was associated with greater perturbations in numbers of circulating lymphocytes, CD4⁺ and CD8⁺ cells. Moreover, caffeine ingestion was associated with an increased percentage of CD4⁺ and CD8⁺ cells expressing the early activation marker CD69 *in vivo* before and after exercise (Bishop *et al.*, 2005a). Furthermore, the post-exercise fall in stimulated neutrophil oxidative burst responses was attenuated by caffeine ingestion (Walker *et al.*, 2006). It is thought that these effects may be largely mediated through the caffeine's action as an adenosine receptor antagonist.

26.5.4 Megadoses of antioxidant vitamins

Vitamins with antioxidant properties, including vitamins C, E and β -carotene (provitamin A), may be required in increased quantities in athletes to inactivate the products of exercise-induced lipid peroxidation. Oxygen free-radical formation that accompanies the dramatic increase in oxidative metabolism during exercise could potentially inhibit immune responses. Sustained endurance training appears to be associated with an adaptive upregulation of the antioxidant defence system; however, such adaptations may be insufficient to protect athletes who train extensively (Powers *et al.*, 2004).

Vitamin C (ascorbic acid) occurs in high concentration in leukocytes and is implicated in a variety of anti-infective functions, including promotion of T-lymphocyte proliferation, prevention of corticosteroid-induced suppression of neutrophil activity, production of IFN and inhibition of virus replication. It is also a major water-soluble antioxidant that is effective as a scavenger of reactive oxygen species (ROS) in both intracellular and extracellular fluids. It can act as an antioxidant both directly (e.g. in the prevention of auto-oxidative dysfunction of neutrophil bactericidal activity) and indirectly via its regeneration of reduced vitamin E.

Studies report that daily supplementation of large doses of vitamin C reduces the incidence of symptoms of URTI in athletes after participation in ultramarathon races (Peters *et al.*, 1993, 1996). The results of one of these studies are illustrated in Fig. 26.4, which also shows that the supplementation of additional dietary

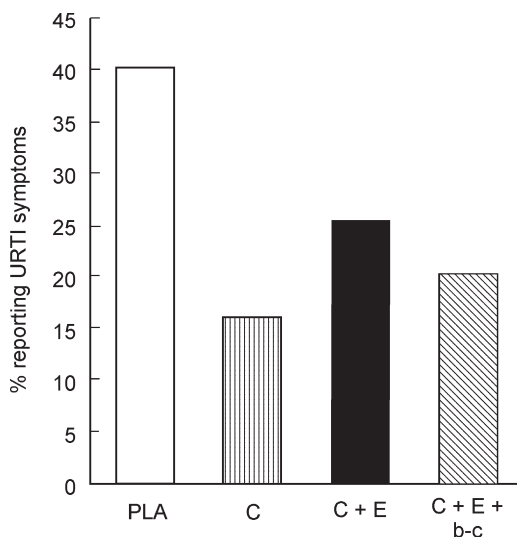


Fig. 26.4 The effect of supplementation with placebo (PLA), vitamin C (C), combined vitamin C + vitamin E (C + E) or combined vitamin C + vitamin E + β -carotene (C + E + b-c) on the percentage of subjects reporting upper respiratory tract infection (URTI) symptoms in the 2 weeks following an ultramarathon race. Data from Peters *et al.* (1996).

antioxidants (vitamin E and β -carotene) does not confer any additional beneficial effect. The doses of vitamin C used in these studies (600–1000 mg/day) are considerably higher than the daily dosage of 200 mg that is needed to saturate body tissues with vitamin C. In a more recent randomised, double-blinded, placebo-controlled study, 1500 mg/day vitamin C for seven days before an ultramarathon race with consumption of vitamin C in a carbohydrate beverage during the race (subjects in the placebo group consumed the same carbohydrate beverage without added vitamin C) did not affect oxidative stress, cytokine or immune function measures during and after the race (Nieman *et al.*, 2002b). In contrast, it has been reported (Fischer *et al.*, 2004) that four weeks combined supplementation with vitamin C (500 mg/day) and vitamin E (400 IU/day) prior to a three-hour knee extension exercise protocol reduced muscle IL-6 release and reduced the systemic rise in both circulating IL-6 and cortisol. Some degree of blunting of the plasma cortisol response to exercise and better maintained neutrophil function after exercise was reported in a placebo-controlled study that used the same dose of daily vitamin C and E supplements and examined immuno-endocrine responses to 2.5 hours of cycling after four weeks of supplementation (Davison *et al.*, 2007). Furthermore, administration of the antioxidant N-acetyl-L-cysteine (a precursor of glutathione) to mice prevented the exercise-induced reduction in intracellular glutathione concentration and markedly reduced post-exercise apoptosis in intestinal lymphocytes (Quadrilatero and Hoffman-Goetz, 2004). Thus, although there are some inconsistencies in the literature regarding antioxidant supplementation and immune responses to exercise, there is some basis for believing that such

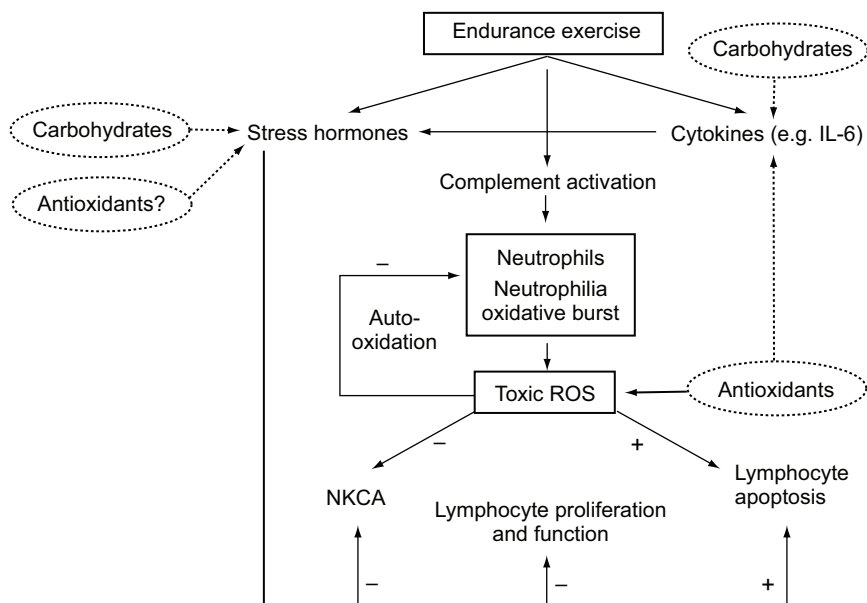


Fig. 26.5 The possible mechanisms by which dietary antioxidant supplementation reduces stress-induced or exercise-induced immunodepression. Also shown is the effect of carbohydrate ingestion during prolonged exercise. NKCA, natural killer cytotoxic activity. Reprinted, with permission, from A. Jeukendrup and M. Gleeson, 2010, *Sport nutrition: An introduction to energy production and performance*, 2nd edn (Champaign, IL: Human Kinetics, page 383).

supplementation could have beneficial effects in alleviating exercise-induced immunodepression via the mechanisms summarized in Fig. 26.5 and Table 26.1.

The most recent Cochrane meta-analysis examined the evidence that daily doses of more than 200 mg vitamin C were more effective in preventing or treating the common cold than placebo (Douglas *et al.*, 2007). Twenty-nine trial comparisons involving 11 077 study participants contributed to this meta-analysis on the relative risk (RR) of developing a cold while taking prophylactic vitamin C. The pooled RR was 0.96 (95% CI 0.92–1.00). A subgroup of six trials that involved physically active subjects (a total of 642 marathon runners, skiers and soldiers on subarctic exercises) reported a pooled RR of 0.50 (95% CI 0.38–0.66). Thirty comparisons that involved 9676 respiratory episodes contributed to the meta-analysis on common cold duration during vitamin C or placebo supplementation. A consistent benefit of vitamin C was observed, representing a reduction in cold duration of 8% (95% CI 3–13%) for adult participants and 13.5% (95% CI 5–21%) for child participants. Fifteen trial comparisons that involved 7045 respiratory episodes contributed to the meta-analysis of severity of episodes experienced while on prophylaxis and the results revealed a benefit of vitamin C when days confined to home and off work or school were taken as a measure of severity. A limited number of trials had examined cold duration and severity during therapy

with vitamin C that was initiated after the onset of cold symptoms, and no significant differences from placebo were found. The authors concluded that the failure of vitamin C supplementation to reduce the incidence of colds in the normal population indicates that routine mega-dose prophylaxis is not generally justified but that individuals subjected to brief periods of severe physical exercise and/or cold environments may well gain some benefit. However, even if some protective effect of high-dose antioxidant supplementation on infection risk is indeed a reality, athletes need to consider the risks that may include the blunting of some of the adaptations to training with a high intake of antioxidants (Ristow *et al.*, 2009), although whether or not this is likely to affect adaptations in already well-trained athletes performing intensive training has recently been questioned (Yfanti *et al.*, 2010).

26.5.5 Vitamin D

Vitamin D is not actually a vitamin but a secosteroid hormone produced in the skin from 7-dehydrocholesterol after exposure to sunlight UVB radiation. The classical role of vitamin D in bone mineralization is very well known, but much less is known about a putative role for vitamin D in maintaining immune function. New insights indicate that sufficient vitamin D is required for the production of antimicrobial proteins (e.g. cathelicidin and defensins) following Toll-like receptor stimulation; as such, vitamin D is purported to play a central role in the anti-bacterial defences that characterize the innate immune response (Laaksi, 2012). Under most circumstances, the major source of vitamin D (~90%) comes from skin sunlight exposure, thus dietary vitamin D (e.g. from oily fish, eggs and fortified cereals) typically accounts for a small component (~10%). However, diet becomes a very important source of vitamin D in northerly latitudes during the wintertime because the limited sunlight exposure is known to be inadequate for inducing endogenous vitamin D production (Laaksi *et al.*, 2010). Adequate skin sunlight exposure to avoid vitamin D deficiency is 5–30 minutes in the middle of the day several times each week (Powers *et al.*, 2011). The Institute of Medicine in the USA have very recently recommended that daily vitamin D intake should be 15 µg (600 IU/day) for skeletal outcomes, but the vitamin D requirements for extraskeletal outcomes such as maintaining immune function remain poorly understood (Ross *et al.*, 2011). Vitamin D status is determined by measuring the serum concentration of the major circulating form of the hormone, 25-hydroxyvitamin D (25(OH)D) which is formed in the liver. Vitamin D deficiency (serum 25(OH)D < 40 nmol/l) is not uncommon and reaches epidemic levels among adults with limited sunlight exposure. Such was the level of concern about vitamin D deficiency in Finland that in 2003 the Ministry of Social Affairs and Health instigated the fortification of milk (0.5 µg/dl) and margarines (10 µg/100 g) with vitamin D: this fortification has substantially improved vitamin D status in Finland (Laaksi, 2012).

Emerging evidence indicates that some athletes also experience vitamin D deficiency. Particularly susceptible appear to be athletes who: live in the northern hemisphere during the winter months; have darker skin (due to the relationship of

skin pigmentation to vitamin D synthesis); train indoors; and, finally, wear protective clothing that covers the skin (Powers *et al.*, 2011). Vitamin D deficiency has been associated with increased infection in a small number of studies. In one study in 756 conscripts serving on a military base in Finland, those with serum 25(OH)D < 40 nmol/l ($N = 24$, 3.6%) experienced significantly more days of absence from duty due to respiratory infection than those with serum 25(OH)D > 40 nmol/l (Laaksi *et al.*, 2007). A follow-up study in 164 military conscripts, by the same group in Finland, showed that supplementation with 10 µg/day vitamin D for six months prevented the typical wintertime fall in serum 25(OH)D (data for $N = 58$) but did not alter the number of days absent due to respiratory tract infection. However, a positive finding was that the proportion of men remaining healthy throughout the six-month period was greater in the intervention group (51%) than in the placebo group (36%) (Laaksi *et al.*, 2010). Clearly, there is a pressing need for studies to determine the efficacy of vitamin D supplementation to improve immunity in athletes

26.5.6 Zinc

Zinc is essential for the development of the immune system and more than 100 metalloenzymes are dependent on it, including those involved in the transcription of DNA and synthesis of proteins. Zinc also functions as an intracellular signal molecule for immune cells (Prasad, 2009). The effects of zinc deficiency on immune function include lymphoid atrophy, decreased delayed-hypersensitivity cutaneous responses, decreased IL-2 production, impaired mitogen-stimulated lymphocyte proliferative responses and decreased natural killer cell cytolytic activity (Chandra, 1984; Overbeck *et al.*, 2008; Prasad, 2009).

Vegetarian athletes are at risk for zinc deficiency because meat and seafood are the richest zinc sources and, although nuts, legumes and whole grains are good sources of zinc, the high levels of fibre in these foods can decrease zinc absorption. Zinc deficiency can also be a problem for athletes in sports where a low body mass confers a performance advantage. Very-low-energy or starvation-type diets may induce significant zinc losses. Because zinc is lost from the body mainly in sweat and urine and these losses are increased by exercise, a heavy schedule of exercise training could induce a zinc deficiency in athletes (Gleeson, 2000).

Studies concerning the relationship between immune function, exercise and zinc status in athletes are lacking. However, megadoses of zinc can actually have detrimental effects on immune function. A study of male runners found that six days of zinc supplementation (25 mg of zinc and 1.5 mg of copper, twice a day) exaggerated the exercise-induced suppression of T-lymphocyte proliferation in response to mitogens (Singh *et al.*, 1984). The administration of zinc (150 mg twice a day) to 11 healthy males for six weeks was associated with reduced T-lymphocyte proliferative responses to mitogen stimulation and impaired neutrophil phagocytic activity (Chandra, 1984). Potential problems with zinc supplements of more than 30 mg per day include nausea, bad taste reactions, lowering of high-

density lipoprotein (HDL) cholesterol, depression of some immune cell functions and interference with the absorption of copper. Hence, large doses of zinc are not recommended. Athletes should be encouraged to consume zinc-rich foods (e.g., poultry, meat, fish and dairy produce). Vegetarians have been advised to take a 10 mg to 20 mg supplement of zinc daily (the RDA is 10 mg and 12 mg for females and males, respectively) but, in view of the above findings, supplements at the lower end of this range may be more suitable for vegetarian athletes (Jeukendrup and Gleeson, 2010).

The efficacy of zinc supplementation as a treatment for the common cold has been investigated in at least a dozen studies published since 1984. The findings have been equivocal, and several reviews of this topic conclude that zinc supplementation may be of benefit in treatment of some diseases such as pneumonia and acute lower respiratory infection but that the results for the common cold are not conclusive (Macknin, 1999; Marshall, 2000; Roxas and Jurenka, 2007; Overbeck *et al.*, 2008; Prasad, 2009). Although only limited evidence suggests that taking zinc supplements reduces the incidence of URTI (McElroy and Miller, 2002; Veverka *et al.*, 2009), in the studies that have reported a beneficial effect of zinc in treating the common cold (i.e. reduction of symptom duration, severity or both) zinc lozenges with high ionic zinc content (>75 mg/day) had to be taken within 24 hours of the onset of symptoms to be of any benefit (Hemila, 2011).

26.5.7 Echinacea and other herbals

Several herbal preparations are reputed to have immunostimulatory effects, and consumption of products containing *Echinacea purpurea* is widespread among athletes. In a double-blinded, placebo-controlled study, the effect of a daily oral pretreatment for 28 days with pressed juice of *Echinacea purpurea* was investigated in 42 triathletes before and after a sprint triathlon (Berg *et al.*, 1998). A subgroup of athletes was also treated with magnesium as a reference for supplementation with a micronutrient important for optimal muscular function. During the 28-day pretreatment period, none of the athletes in the echinacea group became ill, compared with three subjects in the magnesium group and four subjects in the placebo group. Pretreatment with echinacea appeared to reduce the release of soluble IL-2 receptor before and after the race and increased the exercise-induced rise in IL-6.

Numerous experiments have shown that *Echinacea purpurea* extracts exert significant immunomodulatory effects *in vitro*. Among the many pharmacological properties reported, activation of macrophages, polymorphonuclear leukocytes and natural killer cells has been reasonably well demonstrated (Barrett, 2003). Changes in the numbers and activities of T-cell and B-cell leukocytes have been reported but are less certain. Despite this cellular evidence of *in vitro* immunostimulation, pathways leading to enhanced resistance to infectious disease have not been described adequately. Several dozen human experiments, including a number of blind, randomized trials, report health benefits. The most robust data come from trials testing *Echinacea purpurea* extracts in the treatment of acute URTI. Although suggesting a modest benefit, these trials are limited both in size

and in methodological quality. In a randomized, double-blind, placebo-controlled trial, administering unrefined echinacea at the onset of symptoms of URTI in 148 college students did not provide any detectable benefit or harm compared with placebo (Barrett *et al.*, 2002).

In a meta-analysis of trials on echinacea (Linde *et al.*, 2006) that included 22 well-controlled trials, three trials investigated prevention of colds and 19 trials tested treatment of colds. A variety of different echinacea preparations were used. None of the three comparisons in the prevention trials showed an effect of echinacea over placebo. Comparing an echinacea preparation with placebo as a treatment for colds, a significant beneficial effect was reported in nine comparisons, a trend in one and no difference in six. The authors' main conclusions were that echinacea preparations tested in clinical trials differ greatly, there is some evidence that preparations based on the aerial parts of the echinacea plant might be effective for the early treatment of colds in adults but results are not fully consistent. Beneficial effects in preventing colds have not been shown in independently replicated, rigorous randomized trials. Hence, although a great deal of moderately good-quality scientific data regarding echinacea has been gathered, its effectiveness in treating illness or in enhancing human health has not yet been proved beyond a reasonable doubt.

The active ingredients of echinacea extracts are thought to include alkamides, chicoric acid and polysaccharides. Other herbals are also claimed to have various antiviral, antibacterial, immune-modulating and antioxidant properties (for fuller details see Roxas and Jurenka, 2007). Examples include extracts of elderberries (*Sambucus nigra* which contains flavonoids, anthocyanins, glycosides, viburnic acid and vitamins A and C), kaloba (the common name for an extract of the roots of *Pelargonium sidoides* which contains flavan-3-ols, 7-hydroxycoumarin derivatives, proteins and saccharides), ginseng (*Panax quinquefolium* which contains mostly poly-furanosyl-pyranosyl-saccharides), astragalus (*Astragalus membranaceus* which contains polysaccharides, flavonoids, multiple trace minerals and amino acids) and leaves of the olive tree (*Olea europaea* which contains phenolic compounds such as oleuropein and its derivative elenoic acid). Much of the evidence base for these herbal preparations is based on *in vitro* studies demonstrating immune cell stimulatory effects or direct antiviral actions (preventing viral entry into host cells or viral replication). Most preparations like echinacea and others that are classed as 'herbal medicines' are used to reduce the severity and duration of cold symptoms rather than to prevent infections. Whether these are more effective than taking antiviral medications or non-prescription cold remedies containing local anaesthetics, anti-inflammatories, decongestants and stimulants (e.g. ephedrine, caffeine) for the treatment of URTI symptoms such as sore throat, nasal congestion and coughs is debatable.

26.5.8 Polyphenols

Plant polyphenols are potent antioxidant compounds. One of these, quercetin, has received a lot of attention in recent years in relation to its possible effects on

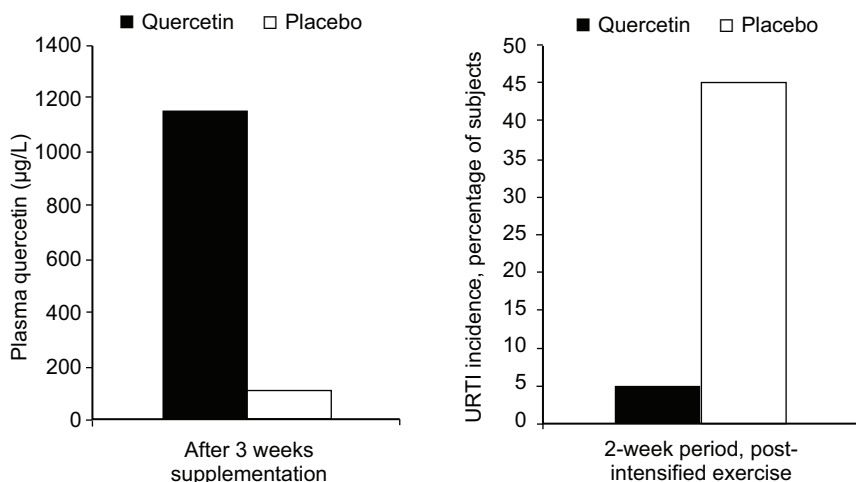


Fig. 26.6 Plasma quercetin levels ($P < 0.001$) and upper respiratory tract infection (URTI) infection rates ($P = 0.004$) in cyclists during a 2-week period following a 3-day period of overtraining and ingestion of quercetin (1000 mg/day) or placebo. Data from Nieman *et al.* (2007).

exercise performance, training adaptation and immune function. Quercetin is classified as a flavonoid, a phytonutrient found in variety of fruits and vegetables. The physiologic effects of these compounds are of great current interest due to their antioxidant, anti-inflammatory, antipathogenic, cardioprotective and anticarcinogenic activities. The richest food sources of quercetin are apples, blueberries, broccoli, curly kale, hot peppers, onions and tea. Total daily flavonol intake (with quercetin representing about 75%) varies between 13 and 64 mg depending on the study sample and the population studied. Human subjects can absorb significant amounts of quercetin from food or supplements, and elimination is quite slow, with a reported half-life ranging from 11–28 hours. Animal studies indicate that seven days of quercetin feeding improves survival from influenza virus inoculation (Davis *et al.*, 2008).

A few human trials have now been conducted and a double-blind, placebo-controlled study with 40 cyclists showed that 1000 mg/day quercetin for three weeks significantly increased plasma quercetin levels and reduced URTI incidence during the two-week period following three successive days of exhaustive exercise (Nieman *et al.*, 2007; Fig. 26.6). In this study a surprisingly high proportion (45%) of subjects in the placebo group reported URTI symptoms in the two-week post-training period, yet markers of immune dysfunction, inflammation and oxidative stress were not different from the quercetin-treated group, suggesting that quercetin exerted direct antiviral effects, at least within the context of the study design. There is increasing support for co-ingestion of quercetin with other flavonoids and food components to improve and extend quercetin's bioavailability and bioactive effects. These include the flavonoid epigallocatechin 3-gallate

(EGCG) from tea, isoquercetin which is the glycosylated form of quercetin in onions and other foods, n-3 PUFA such as EPA and DHA, vitamin C and folate. In a study with 39 trained cyclists, a quercetin supplement combined with EGCG, isoquercetin and n-3 PUFA was more effective than quercetin alone in partially countering exercise-induced inflammation and oxidative stress (Nieman *et al.*, 2009b).

Other naturally occurring polyphenolic compounds are present in foods such green leafy vegetables, onions, apples, pears, citrus fruits and red grapes as well as some plant-based beverages such as citrus juices, green tea, red wine and beer. A recent study investigated whether regular ingestion of non-alcoholic beer polyphenols prior to and after a marathon would attenuate post-race inflammation and decrease the incidence of respiratory illness symptoms (Scherr *et al.*, 2012). Healthy middle-aged male runners ($N = 277$) were randomly assigned to drink 1–1.5 litres per day of a non-alcoholic beer or placebo beverage for three weeks before and two weeks after a marathon race. Blood markers of inflammation were significantly reduced with non-alcoholic beer compared to placebo immediately after the race and 24 hours later and the incidence of respiratory illness was 3.25-fold lower in the non-alcoholic beer group compared with the placebo group during the two-week post-marathon period. Another recent study that examined the effects of regular ingestion of dark chocolate (cocoa is another source of polyphenols) prior to an exhausting bout of cycling reported reduced oxidative stress markers but no effects on hormonal or immune responses to the exercise (Allgrove *et al.*, 2011) so the type and dose of polyphenols may be important.

26.5.9 β -glucans

β -glucans are not only present as major structural components of the cell walls of yeast, fungi and some bacteria, but are also present in the diet as part the endosperm cell wall in cereals, such as barley and oat. β -glucans are carbohydrates consisting of linked glucose molecules and differ in macromolecular structure depending on the source. β -glucans from bacteria are unbranched 1,3 β -linked glycopyranosyl residues. The cell wall β -glucans of yeast and fungi consists of 1,3 β -linked glycopyranosyl residues with small numbers of 1,6 β -linked branches, whereas oat and barley cell walls contain unbranched β -glucans with 1,3 and 1,4 β -linked glycopyranosyl residues. The specific characteristics of the various β -glucans may influence their immune-modulating effects. For example, Brown and Gordon (2003) have suggested that high molecular weight and/or particulate β -glucans from fungi directly activate leukocytes, while low molecular weight β -glucans from fungi only modulate the response of immune cells when they are stimulated (e.g. with cytokines). This implies that the addition of β -glucans to the diet may be used to modulate immune function and so might improve the resistance against invading pathogens in humans.

To date there is limited evidence for immune-promoting effects of orally administered oat β -glucans in animals and humans. Intragastric administration of oat β -glucans in mice enhanced resistance to bacterial and parasitic infections

(reviewed by Volman *et al.*, 2008). Furthermore, Davis *et al.* (2004) demonstrated that daily ingestion of oat β -glucan counteracted the decrease in macrophage antiviral resistance induced by exercise stress in mice. Results from *in vitro* studies from animals treated with β -glucans suggest that β -glucans enhance the immune response in leukocytes and epithelial cells. In the *in vivo* situation, there is now substantial evidence that these effects ultimately translate into an enhanced survival after infection with pathogens (Volman *et al.*, 2008). In this respect, effects are observed irrespective of the β -glucan source and/or route of administration. It has been suggested that the protective effects of orally administered 1,3 β -glucans are mediated through receptor-mediated interactions with microfold cells – specialized epithelial cells for the transport of macromolecules in the Peyer's patches – which lead to increased cytokine production and enhanced resistance to infection. Therefore, it might be possible to modulate immune function by increasing the dietary β -glucan intake, for example by developing functional foods. This may have benefits for specific target populations such as, for example, elderly or type II diabetic patients, but also for athletes involved in heavy training as all of these are characterized by a suppressed (Th1) immune response (Gleeson, 2006a). However, a recent trial in humans found no effect of three weeks of β -glucan supplementation on immune responses to exercise or infection incidence during the two-week period following three successive days of exhaustive exercise (Nieman *et al.*, 2008).

26.5.10 Probiotics

Probiotics are food supplements that contain live micro-organisms which when administered in adequate amounts confer a health benefit on the host. There is now a reasonable body of evidence that regular consumption of probiotics can modify the population of the gut dwelling bacteria (microbiota) and influence immune function (Matsuzaki, 1998; Mengheri, 2008; Borchers *et al.*, 2009; Minocha, 2009), although it should be noted that such effects are strain specific. Probiotics survive gut transit and modify the intestinal microbiota such that the numbers of beneficial bacteria increase and usually numbers of species considered harmful are decreased, which has been associated with a range of potential benefits to the health and functioning of the digestive system, as well as modulation of immune function. Probiotics have many mechanisms of action. By their growth and metabolism, they help inhibit the growth and reduce any harmful effects of other bacteria, antigens, toxins and carcinogens in the gut but, in addition, probiotics are known to interact with the gut-associated lymphoid tissue, leading to positive effects on the innate and even the acquired immune system. This is possible because the gut, as the largest surface area of the body, has a significant role to play in immunity as every day it has to deal with three different immune challenges. First, it must differentiate and tolerate the large commensal microbiota otherwise inflammation will occur, and second, it must also tolerate the food antigens. On the other hand, the gut must be able to mount a defence against any potential pathogens when required. This explains why 85% of the body's lymph nodes are located

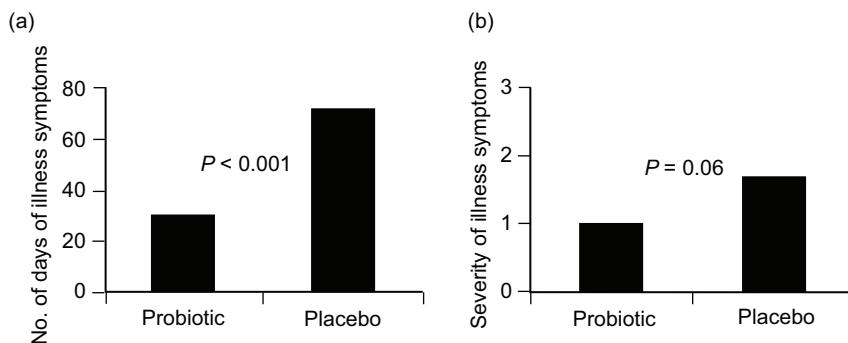


Fig. 26.7 (a) Number of days of illness symptoms for 20 endurance athletes during a month of supplementation with a probiotic or placebo. (b) Mean severity of illness symptoms on the probiotic and placebo treatments. Data from Cox *et al.* (2010).

in the gut, and why probiotics, as functional foods that target the gut, are able to affect the health of the whole body including parts of the body distant from the gut.

Studies have shown that probiotic intake can improve rates of recovery from rotavirus diarrhoea, increase resistance to enteric pathogens and promote antitumour activity. Some evidence even suggests that probiotics may be effective in alleviating some allergic and respiratory disorders in young children (Kopp-Hoolihan, 2001). Although to date there are few published studies of the effectiveness of probiotic use in athletes, interest is beginning to grow, mostly in examining their potential in helping to maintain overall general health, enhancing immune function or reducing URTI incidence and symptom severity or duration (Gleeson and Thomas, 2008; West *et al.*, 2009).

In a double-blind, placebo-controlled, cross-over trial in which healthy elite distance runners received the probiotic *Lactobacillus (L.) fermentum* or placebo daily for 28 days with a 28-day washout period between the initial and the second treatment, athletes ($n = 20$) suffered fewer days of respiratory illness and lower severity of respiratory illness symptoms when taking the daily probiotic (Cox *et al.*, 2010; Fig. 26.7). There was also a significant change in the whole blood culture IFN- γ production, with the probiotic treatment eliciting a two-fold greater change in whole-blood culture IFN- γ production compared with placebo, which may be one mechanism underpinning the positive clinical outcomes. In another study in athletes presenting with fatigue and impaired performance and who had a deficit in blood CD4⁺ (T-helper) cell IFN- γ production compared with healthy control athletes, this apparent T-cell impairment was reversed following a one-month course of daily probiotic (*L. acidophilus*) ingestion (Clancy *et al.*, 2006). Another small study found that consumption of a *L. casei* probiotic yoghurt drink for one month, limited the observed decrease in natural killer cell activity after an exercise stress test (Pujol *et al.*, 2000).

Another probiotic supplement was investigated in 141 marathon runners who were recruited for a randomized, double-blind intervention study in which they received *L. rhamnosus* GG (LGG) or placebo daily for a three-month training

period and then participated in a marathon race with a two-week follow-up of illness symptoms (Kekkonen *et al.*, 2007). Although there were no differences in the number of respiratory infections or gastrointestinal (GI)-symptom episodes, the duration of GI-symptom episodes in the LGG group was shorter than in the placebo group during the training period (2.9 vs 4.3 days) and during the two weeks after the marathon (1.0 vs 2.3 days). In a study on the effect of a *L. casei* probiotic supplement on respiratory tract infection and immune and hormonal changes in soldiers participating in three weeks of commando training followed by a five-day combat course, no difference in infection incidence between groups receiving daily probiotic or placebo was reported (Tiollier *et al.*, 2007). Among the immune parameters investigated, the major finding was a significant decrease in salivary immunoglobulin A (IgA) concentration after the combat course in the placebo group, with no change over time in the probiotic group. A recent randomized, placebo-controlled trial in 64 university athletes reported a lower incidence of URTI episodes during a four-month winter training period in subjects receiving daily probiotic (*L. casei* Shirota) compared with placebo, and this study also reported better maintenance of salivary IgA in the probiotic group (Gleeson *et al.*, 2011). Others have also reported increased levels of salivary IgA (O'Connell *et al.*, 2009) and natural killer cell function (Nagao *et al.*, 2000) in healthy non-athletes after a few weeks of daily *L. casei* Shirota ingestion.

From the research reviewed here, one cannot reach a solid conclusion of probiotic benefit for sportspeople, but there is now sufficient understanding of the mechanism of action of certain probiotic strains, and enough evidence from trials with athletes and sportspeople to signify that this is a promising area of research with mostly positive indications at present. A recent meta-analysis using data from both athlete and non-athlete studies involving 3451 participants concluded that there is a likely benefit in reducing URTI incidence (Hao *et al.*, 2011). Further large-scale studies are needed to confirm that taking probiotics can reduce the number of training days lost to infection and to determine the most effective probiotics as their effects are strain-specific.

26.5.11 Colostrum

Bovine colostrum is the first collection of a thick creamy-yellow liquid, produced by the mammary gland of a lactating cow shortly after birth of her calf, usually within the first 36 hours. Colostrum contains antibodies, growth factors, enzymes, gangliosides (acid glycosphingolipids), vitamins and minerals and is commercially available in both liquid and powder forms. Numerous health claims have been made for colostrum ranging from performance enhancement to preventing infections, but well-controlled studies in athletes are rare. The gangliosides in colostrum may modify the gut microbial flora and act as decoy targets for bacterial adhesion as well as having some direct immunostimulatory properties (Rueda, 2007). A few studies suggest that several weeks of bovine colostrum supplementation can elevate levels of antibodies in the circulation and saliva. In a study of 35 middle-aged distance runners who consumed a supplement of either bovine

colostrum or placebo for 12 weeks, median levels of salivary IgA increased by 79% in the colostrum group after the 12-week intervention, with no change in the placebo group (Crooks *et al.*, 2006). While this result was statistically significant, its physiological interpretation must be viewed with caution due to the small numbers in this study and the large variability in salivary IgA levels. Davison and Diment (2010) reported that four weeks of daily bovine colostrum supplementation prevented exercise-induced falls in salivary lysozyme and speeded the recovery of neutrophil function after two hours of strenuous cycling in healthy men compared with placebo. Further studies are needed to confirm and extend these observations of effects on immune responses to exercise and to establish if bovine colostrum can reduce the incidence of URTIs in athletes.

26.6 Conclusions and recommendations

Both heavy exercise and nutrition exert separate influences on immune function; these influences appear to be greater when exercise stress and poor nutrition act synergistically. Exercise training increases the body's requirement for most nutrients, and in many cases, these increased needs are countered by increased food consumption. However, some athletes adopt an unbalanced dietary regimen and, the poor nutritional status of some athletes very likely predisposes them to immunodepression. Although countering the effects of all of the factors that contribute to exercise-induced immunodepression is impossible, minimizing many of the effects is possible. Athletes can help themselves by eating well-balanced diets that include adequate carbohydrate, protein and micronutrients. Consumption of carbohydrate drinks during training and competition is recommended because this practice appears to attenuate some of the immunosuppressive effects of prolonged exercise. Probiotic and flavonol (e.g. quercetin) supplements may promote some improvement in immune function, but more studies are needed to confirm that taking these supplements can reduce the number of training days lost to infection.

The dangers of over-supplementation of vitamins and minerals should be emphasized because many micronutrients given in quantities beyond a certain threshold reduce immune responses. Other factors that may lower infection risk in athletes include reducing other life stresses, maintaining good oral and skin hygiene, obtaining adequate rest and sleep, avoiding sick people and spacing prolonged training sessions and competitions as far apart as possible.

26.7 Future trends

In vitro cell culture and animal research indicate that advanced supplements such as β -glucan, curcumin, quercetin, isoquercetin, EGCG and other plant polyphenols or unique plant extracts warrant further human investigations to determine if they are effective countermeasures to exercise-induced immune dysfunction and risk of

URTI. The immune system is so diverse that a cocktail of these advanced supplements – maybe as part of a carbohydrate-containing sports drink – will probably perform better than any one alone and recent data support this approach (Nieman *et al.*, 2009b; Bakker *et al.*, 2010). Nieman (2008) has suggested that most benefit is likely to accrue from supplements that primarily target the non-specific, innate arm of the immune system to enhance immunosurveillance against a wide variety of pathogens. Further studies to determine the most effective probiotics are also needed as their effects are strain specific. Research on the effects of vitamin D supplementation in athletes with deficient or inadequate vitamin D status should establish if this practice reduces risk of respiratory infections and/or reduce symptom severity and duration. Larger scale, well-conducted (i.e. randomized, double-blind, placebo-controlled) studies of the effects of nutritional supplements on URTI incidence in athletes are needed as too many previous studies have been low powered and have, with a few exceptions, used only short (days or weeks) periods of intervention. Although various blood or salivary markers of immune function have been measured in human studies examining the mechanisms or efficacy of proposed immune-enhancing supplements, more appropriate markers are probably antibody titre response to influenza vaccination (Albers *et al.*, 2005) and URTI symptom development following controlled nasal rhinovirus inoculation (Schoop *et al.*, 2006).

26.8 Sources of further information and advice

- International Society of Exercise and Immunology
www.isei.dk
The International Society of Exercise and Immunology (ISEI) is devoted to fostering scientific research, education, and the dissemination of scientific information. ISEI provides current practical and valid information to the scientific community based particularly on research directed towards the improvement of health, prevention and treatment of disease through physical activity. The ISEI was founded in 1993 and membership is free. Members get immediate access to information about other members, information about the biannual international meetings organized by ISEI and a free copy of the annual Exercise Immunology Review.
- Gleeson M (2005), *Immune Function in Sport and Exercise*. Edinburgh: Elsevier. A textbook for students of sports science and human physiology. Discusses the acute and chronic effects of exercise on immune function and the strategies that can be used by athletes to reduce their risk of infection.
- Gleeson M (2006), 'Immune system adaptation in elite athletes', *Curr Opin Clin Nutr Metab Care*, 9(6), 659–665.
A review of the impact of acute exercise and heavy training on immune function in endurance athletes.
- Gleeson M (2007), 'Exercise and immune function', *J Appl Physiol*, 103(2), 693–699.

A review of the effects of acute exercise, regular moderate exercise and heavy training on immunity and inflammation.

- Gleeson M, Bishop NC and Walsh NP (2013), *Exercise Immunology*. London: Routledge.

A new textbook for students of sports science and human physiology. Provides an up-to-date review of the acute and chronic effects of exercise on immune function and the strategies that can be used by athletes to reduce their risk of infection.

- Nieman DC and Pedersen BK (2000), *Nutrition and Exercise Immunology*, Boca Raton, FL: CRC Press.

An edited book with chapters written by leading investigators from around the world that review the link between nutrition and immune function, with special application to athletic endeavour.

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27

Malnutrition, immunity and infection

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Abstract: It has been over 50 years since Scrimshaw, Taylor and Gordon set out the bases for interpretation of synergistic and antagonistic interactions between malnutrition and infection. Any contemporary update must recognize emerging diseases such as HIV and re-emerging diseases such as tuberculosis. Genetic and epigenetic concepts and techniques have merged with the advance of technology in basic and applied immunity. Not only nutrient deficiencies, but also their excesses – including obesity and overweight – alter host defenses and risk of infections. Issues of vitamin D, host disease and infection, and interaction of the microbiome of fecal flora with the gut immune system represent two of the exciting contemporary departures.

Key words: infection, nutrition, immunity, stunting, underweight, protein–energy malnutrition, obesity, micronutrient deficiencies, parasites, HIV, emerging infectious diseases.

27.1 Introduction

As the world reaches and surpasses 7 billion inhabitants, 80% still live in low-income and middle-income countries, in which food insecurity, scant health infrastructure and unsanitary hygienic conditions dominate. In sub-Saharan Africa, infections account for the majority of mortality. Elsewhere, the demographic and epidemiologic transitions have moved deaths from chronic diseases into first place. Recent years, moreover, have seen an emergence of new infectious pathogens, such as the human immunodeficiency virus (HIV), and the re-emergence, in association with the latter, of ancient scourges such as tuberculosis, raising the importance of infectious disease in the public health priorities of the entire world (Solomons, 2007). Meanwhile, nutrition transition (Drewnowski and Popkin, 1997), with the supplanting of traditional dietary fare by modern processed foods,

has moved throughout the world over the platform of globalized food trade. Food insecurity at the household level, however, has become a greater public health problem with increasing wealth inequality (Pathak and Singh, 2011) and intermittent inflation of staple food prices (Food Price Watch, 2011).

27.2 The interaction of nutrition, infection and immunity

27.2.1 Historical aspects

The fact that famine and pestilence are two of the four Horsemen of the Apocalypse attests to a long-standing awareness of the association of undernutrition and infection. We can credit the systematic formulation of the paradigm, however, to the trio of Nevin S. Scrimshaw, Carl E. Taylor and John E. Gordon, who combined to write the review paper, 'The interaction of nutrition and infection' in, 1959 (Scrimshaw *et al.*, 1959), and later to produce a contribution to the WHO Monograph series in, 1968 with an expanded report of the same name (Scrimshaw *et al.*, 1968). The fundamental premise of the treatises is that the two domains of illness interact in a manner that is more than the additive sum of the two factors. Interpretation of findings from clinical observations and experimental research provided evidence for *synergism*. Conditions of undernutrition predisposed to increased incidence, duration or virulence of infections of all manner of pathogens known through the 1950s; conversely, acute and chronic infections and infestations predisposed to nutritional deficiency states. To fill out the analysis, the analysts examined the literature for evidence of *antagonism*, as well. Conditions of undernutrition occasionally seemed to be protective for the host, increasing resistance to infection or its consequences. Rare, indeed, were examples in which infections favored acquisition or retention of nutrients. The concepts of the interaction of nutrition and infection were applied in the clinical management of patients, and field trials with interventions with infection control, nutritional improvement or both were launched to inform the public policy implications of the paradigm of Scrimshaw *et al.* (1959, 1968). The history of this paradigm has been placed into clear interpretive context in an analytical historical review by Keusch (2003).

The concept of the interaction of immunity and nutrition is a derivative one. It developed in part from the examination of immune mechanisms in malnourished persons and experimental animals (Sirisinha and Suskind, 1974; Chandra, 1976). Clinical undernutrition was associated with alterations in humoral and cellular nutrition. Nutritionally-induced immunosuppression was accepted as a mediator of the impaired infection resistance observed in malnourished humans and animals. This paradigm was fortified by a burst of interest in the immunoregulatory properties in human milk (Ogra and Orga, 1978; Ogra *et al.*, 1978). It was Scrimshaw again who seized upon developing an understanding of immune mechanisms and regulation and placed it into the interaction format (Chandra and Scrimshaw, 1980; Scrimshaw and SanGiovanni, 1997). It is now generally recognized that immune status represents a sensitive indicator of micronutrient supply.

- Host defense against infection
- The acute-phase (inflammatory) response
- Autoimmunity
- Antineoplastic vigilance

Box 27.1 Classification of the elements of immunity.

Conversely, the activity of the immune system has an effect on the status of and requirements for nutrients (Ströhle *et al.*, 2011).

This author likes to visualize the elements of the immune system in four domains as shown in Box 27.1. There is an important interaction among the elements, especially the second (inflammation) with the first (anti-infection) and the third (autoimmunity). Although the terms ‘increased’ and ‘depressed’ immunity are used in common parlance, the issue is one of appropriate balance and regulation of the responses.

Confirmation of the principles of the interaction of nutrition and infection has come from the epidemiological sphere. It is firmly established that underweight is a co-factor in child mortality from other causes. A substantial percentage of deaths from infectious causes are associated with undernutrition (deficits in weight for age), with the evidence and proportion being defined over two decades (Pelletier, 1994; Caulfield *et al.*, 2004).

There is a clinical colloquial usage for the term ‘immunonutrition,’ which is often used regarding the administration of additional nutrients and sometimes specific supplements such as taurine, arginine or glutamine by enteral or parenteral routes in the perioperative period. This is especially common with extensive oncologic surgery of the abdomen (Mizock, 2010; Klek *et al.*, 2011) or head and neck (Felekis *et al.*, 2010; Bianchini *et al.*, 2012). The concept also applies to burns (Kurmish *et al.*, 2010) and other serious injuries (Khorana *et al.*, 2009). To the extent that there is an evidence base in the practices, they rely on mechanisms related to acute, pharmacological doses of nutrients and dietary components (Quirino *et al.*, 2007; Mühling *et al.*, 2007, 2008; Gunerhan *et al.*, 2009). Exemplifying immuno-modulating nutrients in critical-care settings, Jones and Heyland (2008) comment that ‘*parenteral glutamine is recommended in patients receiving parenteral nutrition, while enteral glutamine should be considered in burn and trauma patients.*’ They continue by stating: ‘*Antioxidants, particularly selenium, should be considered for critically ill patients, and enteral formulas enriched with fish oils are recommended in patients with acute respiratory distress syndrome*’; and finally recommend the term ‘pharmaconutrition.’ These topics are not considered here.

27.2.2 Basic concepts

The viruses, bacteria, fungi and parasites (protozoa and helminthes) embrace categories of single-celled and multicellular organisms that have pathogenic

species capable of infecting and causing disease in humans. Many factors influence the virulence of these organisms, prominent among them the nutritional status of the potential human host. In addition, species that are not usually considered to be 'pathogenic' can produce disease in situations in which the host immune defenses are compromised by virtue of genetic conditions, infections, immunosuppressive drugs or severe undernutrition.

We recognize two domains of nutritional status: (i) those recognized and characterized by abnormal changes in body composition (stature, weight, organ and tissue constitution); or (ii) those related to an insufficiency or excess in the amounts of nutrients required for growth, maintenance or metabolism of the body. These two dimensions are often inter-related. When considering malnutrition in all of its forms (Uauy and Solomons, 2006), which includes deficiencies and overloads, geography and poverty are the prominent determinants of the public health dimension. Nutritional disturbances as secondary result of primary genetic, metabolic or malignant diseases or to the senescence of aging constitute the clinical context.

In general, assessment of body composition-related abnormalities is accessible and straightforward (WHO, 1995; Jaffrin, 2009), whereas assessing the micro-nutrient status of individual patients or free-living populations is fraught with conceptual and technical challenges (Raiten *et al.*, 2011). In the latter instance, the presence of infection or activation of an inflammatory response can complicate the interpretation of biomarkers of nutrient status.

As immune cells are but one metabolically-active component among other tissues, it is not surprising that the general nutritional function supported by nutrients applies to these specialized cells. As pointed out by Maggini *et al.* (2007), vitamins A, C, E and the trace element zinc assist in enhancing the skin barrier function. The vitamins A, B6, B12, C, D, E and folic acid and the trace elements iron, zinc, copper and selenium work in synergy to support the protective activities of the immune cells. They further point out that all of the nutrients, except iron and vitamin C, have a recognized role in the production of antibodies by plasma cells.

27.2.3 Modern departures and recent directions

The genetics of infection susceptibility and resistance and of nutrient handling has been clarified since the resolution of the human genome at the turn of the millennium. Recent years have witnessed increased understanding of epigenetics, as well as mechanisms such as microRNA (Zhou *et al.*, 2011), in the modulation of transcription of genetic information, and their multifaceted role in immunoregulation. Epigenetic regulation may be important for immune responses for both chronic infections (Shin *et al.*, 2011; Stein, 2011) and acute sepsis (Nicodeme *et al.*, 2010; Carson *et al.*, 2011). All of this intricate interaction depends upon signaling mechanisms, and the past decade has revealed some of the heretofore unknown aspects of inter-cellular and intracellular signaling communication (Saric, 2010).

Microbiota and the microbiome, the quasi-immune regulatory apparatus of the intestinal microflora, have potential implications for host defense. Key physio-

logical functions of the commensal microbiota include protective effects exerted directly by specific bacterial species, control of epithelial cell proliferation and differentiation, production of essential mucosal nutrients, such as short-chain fatty acids and amino acids, prevention of overgrowth of pathogenic organisms, and stimulation of intestinal immunity (Tappenden and Deutsch, 2007). It combines with emerging knowledge of the gut-associated immune system of the intestinal wall (Acheson and Luccioli, 2004; Fukatsu and Kudsk, 2011). As stated by Satyaraj (2011) 'Over 65% of the immune cells in the body are present in the gut, making the gut the largest immune organ. Receptors present on the immune cells in the gut are the primary targets for immunomodulation via diet.'

After four decades of a unidirectional focus on undernutrition as the unique public health problem interacting with infection, the last decade has seen a broadening of the question to overweight and obesity (Karlsson and Beck, 2010); this comes in part from a deeper understanding of the inflammatory role of the adipose tissue-derived hormones (adiponectins), specifically of leptin (Fantuzzi, 2006; Gove *et al.*, 2010). Even, in the area of early infant feeding, in which six months of exclusive breastfeeding has been recommended by the World Health Organization for a decade, there is still emergent discovery. The differential impact on the digestive tract of human milk vs infant formula has recently been characterized as an interaction between the metabolic consequences and the effect on the gut microflora (Le Huërou-Luron *et al.*, 2010).

27.3 Growth, body composition and infection

Nutritional conditions based on alterations of growth and body composition have dominated public health attention since the end of World War II, under the generic designation of 'protein–energy malnutrition' (PEM) (although dietary deficiency of neither of these macronutrients fully explains the origin of the syndromes and processes). They are accessible for diagnosis and characterization because only a weighing scale, a tape measure or simply clinical acumen are needed. The earliest classification system was based on deficits in body weight compared to a reference, with Grades I (mild), Grade II (moderate) and Grade III (severe) based on progressive short falls in body mass (Gomez *et al.*, 1958). Clinical differentiation at the severe end of the spectrum included marasmus (inanition), kwashiorkor (edematous) and marasmic kwashiorkor (a combined form). A rating system based on anthropometry for population and public health application was devised by Waterlow (1973), which included abnormal conditions of weight-for-age (underweight), height-for-age (stunting) and weight-for-height (wasting) when the deficits exceeded two standard deviations below the median of a reference population.

27.3.1 Weight-deficit conditions

The classical interest in undernutrition was centered on weight loss and weight deficits. Tuberculosis was termed 'consumption' because of the cachexia produced by the infection. A similar process occurs through the stages of HIV

infection to overt AIDS. Recurrent childhood diarrheal diatheses and respiratory infection episodes can deplete the energy reserves of a child. Although it was originally thought that kwashiorkor arose from a protein-deficient diet, protein intake is likely a conditioning factor, but the appearance of edema in a child with advanced weight loss is often precipitated by a febrile infection such as measles (Torun, 2007). A litany of immune defects was described early in association with severe undernutrition, with edematous forms showing more abnormalities than simple inanition (Suskind, 1980). A more recent synthesis highlights the importance of alterations in the hypothalamic–pituitary–adrenal axis, with a stimulation of glucocorticoids causing thymic atrophy (Cunningham-Rundles *et al.*, 2005). As mentioned, in terms of the synergistic interaction concepts, an overlay of underweight is associated with up to half of the mortality caused by childhood infections (Pelletier, 1994; Caulfield *et al.*, 2004).

27.3.2 Stature deficit conditions

Extreme short stature has been termed ‘chronic undernutrition.’ It is granted that growth-retarded individuals have reduced cell mass and nutrient reserves, compared to peers of normal stature. However, in a proportionality sense, a lower length would oblige a lesser deposit of macro- and micronutrients. The important question for the topic is how and why the linear growth becomes impaired in the first place. Stunting cannot be exclusively or even primarily of dietary origins. Attesting to this is the fact that impaired growth begins in earnest within the first six months of life (Shrimpton *et al.*, 2001), i.e. the period when breastmilk is presumably the substance for most developing country children. Our group and collaborators from the University of California at Davis formulated an argument in favor of futile immunostimulation, induced by abundant, but non-pathogenic microbes in the environment being a major cause (Solomons *et al.*, 1993); this is analogous to the poor growth of poultry and livestock under unsanitary conditions, which is proven to be mediated by a pro-inflammatory cytokine imbalance (Roura *et al.*, 1992). Recent findings from Nepal, linking intestinal mucosal damage to systemic immunostimulation to growth failure links the inflammatory mechanism back to the human context (Panter-Brick *et al.*, 2009). More specific mechanistic work related to the skeletal elongation defects has recently come from a protein-modification and immunostimulation mouse model, in which specific influences on the hormonal cascade for epiphyseal enlargement are being delineated (Odiere *et al.*, 2010).

There is epidemiological evidence that recurrent episodes of childhood infections predispose to short stature. *Giardia intestinalis*, a protozoal parasite, which colonizes the upper intestine and occasionally erupts into symptomatic enteritis, is an interesting case in point. All three studies on the association of giardiasis with linear growth in Guatemala have shown a negative interaction (Farthing *et al.*, 1986; Cook *et al.*, 2009; Duffy T, unpublished findings, 2010); this was true of a recent inquiry in Colombia as well (Boeke *et al.*, 2010). Studies in other settings,

however, have found no relationship (Lunn *et al.*, 1999; Campbell *et al.*, 2004; Hollm-Delgado *et al.*, 2008). The sensitivity of the methods to quantify the protozoa might explain the discrepancies. Also, intestinal helminthic parasites and recurrent infections with *Helicobacter pylori* have been implicated in linear growth impairment for children beyond the infant and toddler years. The findings for both classes of organism are widely divergent, however, from population to population, indicating interactions with other modulating factors to explain the inconsistent reports. Finally, exposure to the fungal mycotoxin, aflatoxin, seems to play a role in height faltering (Khlangwiset *et al.*, 2011).

Stunting is recognized to predispose one to deficits in performance, including in resistance to disease (Rah *et al.*, 2010). Information, however, is relatively sparse regarding specific immune responses differing in stunted children compared to children of normal stature in the same environment. One clear and relevant example, however, relates to malaria. It has been established that stunting predisposes to more severe complications of malaria parasitemia, namely anemia (Verhoef *et al.*, 2002). With respect to immune mechanisms, in Senegal, stunting was associated with lower anti-*Plasmodium* antibody responses as compared to children of better linear growth status (Fillol *et al.*, 2009).

27.3.3 Obesity and overweight conditions

One and a half billion inhabitants of the world are estimated to be overweight or obese. Moreover, it is not only the affluent who suffer; poverty is seen as a strong risk factor for excessive weight, because quality foods are beyond the means of the poor. More than just a simple imbalance between energy consumption and expenditure, there are apparently genetic and epigenetic issues. The notion of natural selection for a 'thrifty gene' (by modern day standards a complex of polymorphisms) is predicated on the evolutionary idea that ability to acquire and retain energy favored fitness through millennia of alimentary insecurity and feast and fast cycles. Closely aligned is the evolutionary hypothesis that efficient energy acquisition and retention in human evolution had advantages for individual survival over the short lifespan. An assertion of the trade-off by Roth and collaborators (2011) is quoted in Box 27.2. With pharmaceutical and vaccination control of infections and a longer survival, the benefits for early adult life turn to liabilities as the years progress. At the epigenetic regulation level, adherence to the developmental origins and fetal programming school refer to a 'match-mismatch' mechanism, in which the efficiency for energy and nutrient retention is governed by environmental messages transmitted to the mother and possibly engraved by epigenetic mechanisms (Godfrey *et al.*, 2007); if the maternal environment signals scarcity, the phenotype will express energy efficiency.

Recent advances in technology, including genetic identification of bacteria, have advanced the study of gut microbiology. At the same time, studies are revealing an important role of the intestinal flora in overall energy regulation (Cani *et al.*, 2008). In terms of putative mechanism, Delzenne and Cani (2011) comment:

... an immune system that is often up-regulated by nutrition-related signal, independent of the actual presence of a pathogen. We propose that an overactive immune system, including the 'metabolic syndrome', was adopted evolutionarily in the distant past to help hold out against unconquerable infections such as tuberculosis, malaria, and trypanosomiasis. This immune activation is advantageous in the absence of other disease management methods, especially under conditions in which life expectancy is short.

Source: Roth *et al.* (2011).

Box 27.2 Commentary on the association of human immune regulation and the evolution of energy metabolism.

'Animal and human data demonstrate that phylogenetic changes occur in the microbiota composition in obese versus lean individuals; they suggest that the count of specific bacteria is inversely related to fat mass development, diabetes, and/or the low levels of inflammation associated with obesity.' An elegant prospective study, involving prospective overfeeding of 1000 kcal daily in lean and obese individuals, revealed a difference in the species makeup of the intestinal flora of the two body types but, more importantly a differential response of the microbiota to the intervention (Jumpertz *et al.*, 2011). The flora of the lean individuals proliferated in a way that could account for an increased energy degradation by the gut organisms, whereas the flora of the obese failed to increase its dissipation of energy. The intestinal flora are now confirmed as another 'organ' of energy regulation for the body.

Karlsson and Beck (2010) have recently produced a comprehensive review of infections in overweight conditions. They review immune studies in diet-induced and genetically-derived obese rodent models and find the documentation of abnormalities of function of macrophages, T and B cells, null killer (NK) cells and dendritic cells. Obesity has an adverse effect on immunological memory, manifested both in the efficacy and duration of vaccine-induced immunity and on the function of memory T cells. In both experimental infections in animals and in infection epidemiology for humans, excess body fat corresponds to poorer clinical experience with specific infectious agents. In humans, the litany includes viruses such as influenza and coxsackie and bacteria including *Mycobacterium tuberculosis* and *Helicobacter pylori*. In the clinical and surgical context, this interaction can be seen in a study of nearly 600 elective surgery patients, in which percent body fat, estimated by bioelectrical impedance, was associated with post-operative surgical site infection; frank

obesity (a body mass index >30 kg/m²) raised the wound infection risk five-fold (Waisbren *et al.*, 2010).

With respect to the physiology of obesity, it constitutes a 'low-grade inflammatory state' (Wärnberg and Marcos, 2008), due to the production of adipose-derived polypeptide hormones (adiponectins) which, in turn stimulate pro-inflammatory cytokines. Fernández-Riejos *et al.* (2010) go even further, stating: 'Adipose tissue is an active endocrine organ that secretes various humoral factors (adipokines), and its shift to production of pro-inflammatory cytokines in obesity likely contributes to the low-level systemic inflammation that may be present in metabolic syndrome-associated chronic pathologies such as atherosclerosis.' Thus, leptin and its adipokine cousins are potential upstream actors in processes that can alter the course of the response to infectious pathogens.

27.4 Selected micronutrients

To the extent that innate and adaptive immune responses depend on intact somatic cells and barrier tissues, severe deficiency of any micronutrient capable of disrupting cellular integrity will debilitate the basic immune response. This review seeks a more particular and mechanistically-based approach. The interactive issues relate to infections provoking deficiencies (or excesses), deficiencies (or excesses) affecting infectious risk, and deficiencies (or excesses) impairing immune regulation. Deficiency of some nutrients, such as biotin, pantothenic acid, copper and manganese, among others, does not have manifestations within the general population, but only in extreme clinical circumstances. It has been impossible to examine nutrition–infection biology in these rare instances. On the other side of the ledger, excessive intakes of some nutrients are virtually unachievable within common dietary or supplementation practices or, as in the case of vitamin B12, produce no recognized adverse effects at any dosage. Hence, the review addresses selected nutrients with public health importance for either the deficiency or the overload situations.

Extensive reviews on some or all aspect of the interaction exist, including in publications over the last decade (Bhaskaram, 2002; Field *et al.*, 2002; Louria, 2007; Solomons, 2007; Wintergerst *et al.*, 2007). Since this review is focused on the most recent insights into the nutrition–infection–immunity questions and conundrums, it will only refer cursorily to long-established information in order to introduce and highlight important discoveries and insights gathered during the past decade of investigation.

27.4.1 Vitamin A

It is well established that many infections, including measles and invasive diarrheas, produce vitamin A deficiency. The emerging infection of HIV (Kassu *et al.*, 2007; Papathakis *et al.*, 2007) and tuberculosis (Mathur, 2007) are associated with hypovitaminosis A. In a cohort of HIV-infected patients in Boston under control

with highly active anti-retroviral therapy (HAART), low retinol levels were uncommon (Jones *et al.*, 2006). Vitamin A was once termed the ‘anti-infective’ nutrient based on work in experimental animals and livestock (Underwood, 1994). Numerous intervention trials were conducted in the 1980s and 1990s; they confirmed that periodic supplementation with vitamin A reduces mortality from common childhood infections. Careful examination of the experiences reveals, however, that the *incidence* of infections did not differ from the untreated subjects. As such, it is concluded that enhanced vitamin A status acts by reducing the severity and lethality of infections, but does not prevent contagion. Experience in India suggests vitamin A supplementation can be adjunctive to treatment of tuberculosis (Mathur, 2007), although no systematic trials have been published.

Results from major vitamin A supplementation trials have been evaluated to see how they might inform public policy. With regard to universal supplementation of mothers, a meta-analysis focusing on clinical infections showed suggestions of a protective effect, including a reduction in maternal infections (van den Broek *et al.*, 2010). However, in areas endemic for HIV transmission, important and contrasting interactions have been seen. Vitamin A supplementation to HIV-infected infants seems to be protective for their growth (Villamor *et al.*, 2005), but a complex relationship among vitamin A regimens (supplementing mothers only, supplementing neonates only or supplementing both) vs placebo was found (Humphrey *et al.*, 2006). None of the regimens interrupted maternal-to-child-transmission. Supplementation only to the neonates improved early survival; by the six-month follow-up, however, all forms of vitamin A administration were associated with twice the mortality as that seen in the no-treatment group. Others studies have explored vitamin A supplementation among developing-country mothers themselves; oral vitamin A had both positive and negative consequences for aspects of the reproductive cycle (pregnancy, birth, lactation) in African women infected with the virus, with the notable disadvantage of promoting vertical transmission of the virus from mother to offspring (Mehta and Fawzi, 2007).

New insights on the consequence of vitamin A status on infection come from studies in the Northeast of Brazil (Lima *et al.*, 2010) in which oral vitamin A supplementation reduced giardiasis burden, especially via reduction of new infections. Excess intake of vitamin A can produce toxicity manifestations involving the liver and central nervous system, but no specific consequences relating to immunity or infection risk have been attributed to this.

27.4.2 Vitamin D

The first decade of the millennium has seen the greatest intensity of new revelation in the area of vitamin D and immunity. The degree to which the vitamin influences human host defenses and infection experience is of primary interest here. Interestingly, since humans depend primarily on solar exposure to support vitamin D nutriture, there is scant documentation of infectious processes contributing directly to exacerbating risk of hypovitaminosis D status and rickets. Walker and

Modlin (2009) comment: 'Recent epidemiologic studies clearly demonstrate the link between vitamin D deficiency and the increased incidence of respiratory infections.' Pulmonary tuberculosis is the primordial respiratory infection, and incidence has resurged in the era of HIV. One report on patients with HIV complicated by tuberculosis imputes increased risk of vitamin D deficiency, the underlying inflammatory processes of both infections however were not associated with the vitamin D status (Friis *et al.*, 2008). Without specific supplementation with the vitamin, circulating 25-hydroxy vitamin D levels increased by 11% in tuberculosis patients in northern Tanzania during the first two months of treatment, although increased appetite for food and improved mobility may have improved the dietary intake and sun exposure (Tostmann *et al.*, 2010).

The association of HIV, *per se*, and vitamin D was addressed by Villamor (2006) who points to 'An inverse association between 1,25(OH)₂D concentrations and mortality has been reported from a small cohort of HIV-infected adults, and some cross-sectional studies have indicated positive correlations between 1,25(OH)₂D and CD4⁺ cell counts.' A research group in the Netherlands (van den Beukel, 2008) identified a group of vitamin D-deficient patients in their clinics for treatment of HIV, and administered 2000 IU (50 µg) of vitamin D daily for 14 weeks, and 1000 IU (25 µg) for an additional 34 weeks, with no control group. The positive effects of increased levels of both 25-hydroxy and 1,25-dihydroxy vitamin D and suppressed parathyroid hormone were seen in the initial period, but on the halved dose only an elevation of 25-hydroxy vitamin D persisted at 48 weeks. In the initial phase, however, the vitamin D supplementation induced decreased insulin sensitivity, which also normalized with the dosage decrease. No information on the parameters of HIV infection was given.

As to the converse side of the issue, reduced vitamin D status appears to have implications for resistance to infection. Consequences of vitamin D deficiency on immunity relate to impaired T-lymphocyte function and interleukin (IL)-10 production (Ginde *et al.*, 2009), as well as weakening of the innate immunity in the barrier functions of skin and mucosal epithelia (Schwalfenberg, 2011). Best defined to date on a mechanistic basis is the putative role for vitamin D in defense against *Mycobacterium tuberculosis* (Ginde *et al.*, 2009; Yuk *et al.*, 2009; Schwalfenberg, 2011). The putative mechanism is vitamin D's role in the expression of human cathelicidin, which is an 18-peptide antimicrobial peptide (Ginde *et al.*, 2009); it has special action against the tuberculosis pathogen, by inducing autophagy by monocytes which have ingested the bacterium, by attracting phagosomes, helping to assure its lysis and destruction of its content (Yuk *et al.*, 2009). The agenda for the immediate future with respect to vitamin D and infections, according to Taylor and Camargo (2011), is to deepen our understanding of vitamin D receptor genetics on the host and pathogen side, while planning appropriately designed randomized controlled trials of vitamin D supplementation in groups at high risk for infection, including infants, children and the immunocompromised. Finally, there is a vigorous debate on what upper tolerable limits for vitamin D really are and how high the dose must go in order to produce excessive dosages. No negative effects on immunity from high exposures to the vitamin are yet recorded.

27.4.3 Vitamin E

Low vitamin E status can be a consequence of uncommon digestive disorders such as cystic fibrosis. HIV was associated with low α -tocopherol in lactating women in South Africa (Papathatkis *et al.*, 2007), but not in Bostonians on HAART. Deficiency can be a problem in neonatal life, but it is not considered to be a public health problem for the remainder of the free-living population. As such, infectious exposure is not a major determinant of interest for nutritional status.

There is, however, an opposite narrative for experimental vitamin E deficiency and resistance to at least one infection: malaria. Older findings explored the ability for vitamin E-deficient diets to prime red cells for susceptibility to oxidative stress; once the deficient erythrocyte was invaded by a *Plasmodium*, it collapsed and was removed by the spleen before the protozoa could proliferate and extend to parasitemia (Levander and Ager, 1993). Nutritional deficiency of the host with a protective outcome is what has become widely known as 'nutritional immunity' (Weinberg, 1999). In a mouse model, vitamin E deficiency decreased resistance to experimental helminth infection (Au Yeung *et al.*, 2005), however the deficiency also protected the host animals from adverse metabolic and intestinal permeability consequences of the parasitosis.

27.4.4 Vitamin C

Scurvy is a malady of legend, with the historical lore as an affliction of sailors on the high seas or prisoners in internment camps. It can occur in eating disorders and alcohol abuse, but scurvy is rarely seen in clinical practice. Biological evidence over the past decades has progressively *reduced* estimates of daily dietary requirements for ascorbic acid. Specific infections inducing vitamin C deficiency have not been catalogued. As a member of the class of 'antioxidant' micronutrients, this vitamin has often been affiliated, by extension, with immunity (Wintergerst *et al.*, 2007; Maggini *et al.*, 2007), but recent evidence is sparse. Two recent publications related to programmed cell death (apoptosis) are of note. Pavlovic and Sarac (2010) document the evidence for a role of ascorbic acid in apoptosis of thymocytes. Specifically related to malaria, apoptosis in cultured vascular endothelial cells *in vitro*, mediated by the sera from patients with severe malaria, was reduced by addition of ascorbic acid to the medium (Hemmer *et al.*, 2005).

27.4.5 Folate

Except for regions dependent upon boiled maize as the primary dietary staple, endemic folate deficiency leading to macrocytic anemia as a widespread manifestation is rare. The amount of folate that individuals are recommended to consume, however, has doubled within the last decade, with specific considerations of prevention of neural tube defects (Simpson *et al.*, 2010). Hence, individuals who were consuming 'sufficient' amounts of folates by earlier standards may now show deficient intake in contemporary dietary surveys. Infections are only tangentially involved in determining folate status. HIV infection in South Africa, a country

dependent upon maize meal as the principal staple, is associated with folate deficiency (Papathatkis *et al.*, 2007). Malaria has a complex relationship with folate nutriture and metabolism, with some studies ascribing a depleting role in pregnancy (Chango and Abdennebi-Najar, 2011).

Microbial toxins or actions are believed to be involved in the etiology of tropical enteropathy, an intestinal malady of reduced digestive and absorptive efficiency of the small intestine, affecting populations living in unhygienic conditions in tropical climes (Ramakrishna *et al.*, 2006; Humphrey, 2009); folate deficiency has been associated with these enteropathic disorders, and tetracycline plus folic acid is one of the treatment regimens used to relieve the condition (Kumar *et al.*, 2011).

Malaria is the febrile consequence of red cell invasion by species of the *Plasmodium* protozoa. Folate is a co-factor needed for the malaria parasite's growth and reproduction. The parasite depends on intraprotazoal metabolism of folates; many antimalarial drugs are based on inhibiting the enzyme, dihydrofolate reductase, within the parasite (Hyde, 2002; Carter *et al.*, 2005). Thus it has been debated inconclusively to what extent the folate status of the human host or oral ingestion of folate compounds relates to the successful infection of our species (Chango and Abdennebi-Najar, 2011).

Folic acid (pteroyl glutamic acid) is a synthetic form of the vitamin, only found in minor amounts in foods or tissues. It is now added increasingly to staple grain flours, breakfast cereals and snacks. It has recognized immunomodulation with respect to carcinogenesis (Sanderson *et al.*, 2007), which could imply some immune consequences for anti-infective host defenses. Beyond the malaria narrative, however, there are few clear examples. To the extent that dysplasia and more advanced neoplastic changes of the uterine cervix are related to a viral infection, that with human papilloma virus (HPV), the putative relationship with dietary folate exposure is relevant to this theme. According to Piyathilake (2007), the uncertainties about the relationship had to do with incomplete validity in both diagnosis of the cervical lesion and exposures of subjects to folate. He asserts that '*recent studies that addressed these issues have demonstrated that folate is an important micronutrient in cervical cancer prevention via its influence on HR-HPV and the development of CIN (cervical intraepithelial neoplasia).*'

27.4.6 Vitamin B12

Classically, vitamin B12 deficiency has been associated with a very specific intestinal helminth, that of the fish tapeworm, *Diphyllobothrium latum*. HIV infection has recently been associated with vitamin B12 deficiency (Papathatkis *et al.*, 2007). Conditions of malabsorption would be candidates to impair nutrient status. As *H. pylori* infection can exhaust gastric mucosal function, and affect intrinsic factor secretion, attention has recently been focused on evidence suggesting vitamin B12 deficiency associated with that bacterium (Amarapurkar and Amarapurkar, 2010; Anon, 2011). Surprisingly, there is no recent or remote knowledge on any specific compromise of immune function and increased susceptibility to disease, despite the well-known morphological alterations of multilobular

polymorphonuclear leukocytes and macrocytic lymphoid cells in megaloblastic bone marrow processes.

27.4.7 Magnesium

At least 60% of US residents do not consume the recommended daily amounts of magnesium considered as an adequate intake by the Institute of Medicine, but adverse consequence are not commonly recognized (Nielsen, 2010). Assessment of magnesium status is limited by the absence of reliable biomarkers (Arnaud, 2008). With such a tenuous handle on nutritional status, it is not surprising that no direct interactions of infections and magnesium status have been resolved, although severe diarrheal and vomiting diatheses due to gastrointestinal pathogens can produce transient hypomagnesemia. A more insidious effect on magnesium status may result from chronic low-grade inflammation, as in the example of obesity (Nielsen, 2010). Since many chronic viral, bacterial and parasitic diseases produce such an inflammatory response, analogous depletion could be an as yet unrecognized consequence of HIV, tuberculosis or malaria. In an experimental rodent model, dietary magnesium deficiency increased both the basal and stimulated activity of neutrophilic polymorphonuclear leukocytes, with the cells showing increased metabolic activity and phagocytosis. This was reflected in the whole animals as elevated IL-6, higher nitric oxide concentrations and increased lipid peroxidation (Bussière *et al.*, 2002). The implications for response to human infections are unknown.

Hypermagnesium causes cardiac irritability. It is rarely seen except in an iatrogenic context or with self-medication of magnesium salts, especially in aging and renal insufficiency settings. Immunological consequences are not described.

27.4.8 Iron

Blood-feeding intestinal helminths and protozoa and recurrent bouts of dysentery are known to produce iron deficiency through fecal blood loss. *Schistosomiasis hematobium* can produce a similar effect through hematuria. The exhaustion of gastric acid secretion after chronic recurrent *H. pylori* infections represents another factor, acting through reducing dietary iron intake. Respiratory and diarrheal infections did not predispose to iron deficiency in one study in Pakistani toddlers (Ali and Zuberi, 2003).

It is also a long-established fact that iron deficiency impairs a series of host defense mechanisms from phagocytosis to antibody formation. Evidence is mixed, however, on whether iron deficiency increases risk for the incidence of human infections. Recent years have witnessed new observations on the effects of iron deficiency as a predisposing factor to infections. A longitudinal study of Bedouin families in Israel (Levy *et al.*, 2005) showed early-life anemia, presumed to be due to iron deficiency, to be an independent risk factor for diarrhea and respiratory illness from seven to 18 months of age. In a comprehensive study in menstruating French women, no excess infection could be attributed to iron depletion (Duport *et*

al., 2003). Of course the environmental burden of pathogens is far lower in France than in tropical regions. However, again in a developed European country, Belgium, iron deficiency without anemia was a strong risk factor for bacterial vaginosis in early pregnancy (Verstraelen *et al.*, 2005). In tropical, developing countries, in which the environment provides numerous sources of inflammation, the interpretation of the biomarkers of iron status such as zinc protoporphyrin, ferritin and soluble transferrin receptors is confounded (Friis *et al.*, 2009); specific algorithms may help to correctly detect iron deficiency in the presence of inflammation.

The most widely regarded issues of iron deficiency for the immune system relate to impaired cellular immunity, as related to T-lymphocyte proliferation studies. A new examination of established immune parameters has been undertaken in Bolivia (Sejas *et al.*, 2008), to confirm the phenomenon and assess the reversibility of the defect. Indeed, iron-deficient schoolchildren had lower circulating counts of CD1a⁺ T-lymphocytes as compared to iron-replete controls. Three months of intensive oral iron supplementation improved the circulating counts of the former group, but they still remained depressed compared to normative control levels.

In their treatise ‘Nutritional iron deficiency: an evolutionary perspective’, Denic and Agarwal (2007) comment that: ‘Multiple evolutionary factors have contributed in making iron deficiency a successful phenotype.’ They suggest that the emergence of primitive agriculture caused an aggregation of populations with consequent increase of communicable diseases while simultaneously reducing dietary iron availability and that the latter adaptation acted to mitigate the virulent effects of the former. This evolutionary theory supports both the theoretical and empirical bases of the concept of ‘nutritional immunity’ first enunciated by E.D. Weinberg in the 1970s, and reviewed in 1999 (Weinberg, 1999).

The site of deprivation of pathogens from iron could be in the circulation or in the iron stores, depending on the nature of the host–microbe interaction. Adverse results in a large field trial on oral iron and folic acid supplementation to anemic children in a malaria zone of Tanzania, on the Island of Pemba (Sazawal *et al.*, 2006), brought iron at the point of administration into profound relief. The iron supplementation had to be curtailed prematurely when the incidence of hospitalization and death of those receiving iron exceeded that of the placebo and zinc-only arms. It has been speculated that the flux of loosely-bound iron following the iron supplementation is a factor in the aggravation of malaria, as the children with adequate iron stores at the time of entry into the study were the most severely affected. Whether iron deficiency is protective or detrimental to host response to malaria has been debated for decades. In a very recent study from Malawi, women with acute or acute and chronic placental malaria were 60% less likely to be iron deficient, evoking renewed suggestion for operation of nutritional immunity (Senga *et al.*, 2011).

As the HIV has an iron requirement, the notion of a nutritional immunity issue with this infection has been raised. In a study among Tanzanian women monitored for progression of their HIV-status with careful staging, no gradient relationship of

clinical stage with serum ferritin was observed, although concentrations above 150.0 µg/L were related to a non-significantly elevated risk of progression to stage 4 (Kupka *et al.*, 2007). By contrast, in a cohort of non-anemic Zimbabwean women, a serum ferritin concentration above 45 µg/L was associated with significantly higher HIV viral load and 12-month mortality rates (Rawat *et al.*, 2009). A prospective four-month randomized clinical trial of low-dose iron (60 mg of iron daily) in anemic pregnant HIV-positive African women failed to produce any change in circulating viral loads (Olsen *et al.*, 2004). They note that, for ethical reasons, a *cautiously* low dose was given, and speculate that adverse effects may occur when seropositive women are exposed to the more typical 60 or 120 mg daily iron prenatal regimens.

27.4.9 Zinc

Based on the prevalence of short stature and the low biological availability of zinc in whole grain staples across developing societies, primary zinc deficiency is believed to be one of the most widespread nutritional deficiencies worldwide. The absence of a reliable diagnostic biomarker, however, limits the epidemiological estimates (Raiten *et al.*, 2011). There are abundant mechanisms for secondary (conditioned) deficiency including intestinal malabsorption disorders, excessive secretion of digestive fluids and increased urinary loss. Zinc was not featured prominently in the Scrimshaw *et al.* (1959) review as secondary human zinc deficiency, as described by Vallee *et al.* (1959) was in the same year. Over the past half century, however, zinc deficiency has emerged over the years as a consequence of numerous infections both acute and chronic. Most recently, low zinc levels have been found in HIV infected individuals in numerous settings (Jones *et al.*, 2006; Papathakis *et al.*, 2007; Ndeezi *et al.*, 2010).

Conversely, clinical zinc deficiency states predispose to impaired resistance to infectious disease. As a consequence, from the 1970s through the 1990s, research into the effects of experimental zinc deficiency on human and animal immune mechanisms dominated that for any other essential nutrients. The resultant inquiry identified defects in almost all aspects of innate and acquired immune systems (Fraker and King, 2004). One of the most recently identified aspects of zinc's involvement in immune function has been with the ameboid microglia cells, which are part of the immune-defense system of the central nervous system. Manipulation of the extracellular zinc milieu *in vitro* and in intact animals moderated the release of matrix metalloproteinases, reactive oxygen species and other pro-inflammatory factors characteristic of this cell line (Kauppinen *et al.*, 2008). Excess administration of zinc can induce copper deficiency, which lowers white cell counts; this is without implications for infectious risk as reviewed below.

Recent field trials have demonstrated that oral zinc supplementation can prospectively reduce the rates of diarrhea and respiratory infections. The findings are not consistent, however. As an example, Luabeya *et al.* (2007) in South Africa failed to find any additional protection from these two illnesses with daily administration of 10 mg of zinc to children either alone or as part of a multivitamin intervention

regimen. On the other hand, from a therapeutic point of view, the ability of zinc to curtail severity and duration of diarrhea episodes has been amply demonstrated (Lazzerini and Ronfani, 2008). The WHO recommends a daily dose of 20 mg of elemental zinc as an adjunct to oral rehydration therapy for diarrhea. The action of zinc in this context may or may not be related to any pre-existing deficiency of zinc.

Activation of the acute-phase response leads to sequestration of zinc in the liver, acutely lowering zinc levels. Recently, Kehl-Fie and Skaar (2010) have invoked the notion that this response has evolutionary protective effects of 'nutritional immunity' analogous to that with iron, by depriving pathogens of access to the nutrient. Confirmatory evidence remains to be developed.

27.4.10 Selenium

Both HIV and hepatitis C infections among Argentine drug users resulted in impaired selenium status (Sheehan *et al.*, 2011). Conversely, selenium status did not suffer among North American HIV-infected adults under HAART control (Jones *et al.*, 2006). Keshan disease, an enigmatic pulmonary disease with potentially an infectious origin endemic to low-selenium regions of China, has long been related to impaired selenium intakes as a co-factor (Xu *et al.*, 1997).

In a cohort of HIV-positive Tanzanian mothers, six months of daily supplementation with 200 µg of selenium as selenomethionine produced a 40% reduction in diarrheal incidence, but had no effect on any other morbidity indicators (Kupka *et al.*, 2009). However, somewhat older and well-disseminated experiments out of the laboratory of Dr Melinda Beck and colleagues (2004) have illustrated a theretofore unknown scenario of the nutritional status of the host animal, specifically depleted or repleted in terms of selenium, altering the phenotype of a murine coxsackie virus, toward a permanent state of virulence. The native strain of the virus was innocuous to well-nourished mice, but produced a fatal lung infection in selenium-deficient animals (Beck *et al.*, 2004). The virus recovered from the deceased animals and passed into healthy, selenium-sufficient mice had acquired a virulence factor which made it lethal to well-nourished hosts.

Regarding the issue of the consequence of selenium deficiency on immunity, nutritional immunity examples predominate. In the aforementioned study on murine helminthiasis (Au Yeung *et al.*, 2005), selenium deficiency was also induced, producing decreased resistance to infection by the worms, but none of the other protective effects shown by vitamin E depletion. Lack of any protective effect has been observed in other experimental parasite models (Hoffmann and Berry, 2008). Excessive selenium accumulation in the body is common in high seleniferous zones of China and other countries, but consequences of selenium excess on infectious risk or immunity are unknown.

27.4.11 Copper

Although copper intakes may not meet recommended levels, after early infancy, clinical copper deficiency is almost completely unknown in free-living

populations. Infections play little role in copper status. Due to environmental contamination, such as copper pipe and vessels, the more common disorder of copper nutrition in humans is excessive oral intake of the metal (Muñoz *et al.*, 2007), although no adverse consequences on immune function have been documented. Leukopenia is a long-recognized consequence of experimental and clinical copper deficiency, but ability to mount an appropriate leukocytosis with infectious challenges has generally been shown to be intact. Delayed hypersensitivity, as measured by lymphocyte stimulation reactions to various mitogenic stimuli, was constant throughout a range of dietary intake from adequate to deficient (Rhee *et al.*, 2004).

27.4.12 Chromium

Chromium depletion has generally been associated with diabetes and other disorders of glucose tolerance, but infections are not widely regarded to stress the chromium reserves of humans or animals. Clinical chromium deficiency has only been seen in iatrogenic situations, and does not exist as a public health problem. Without support from a biomarker of chromium status other than a degree of glucose intolerance, a randomized trial of chromium supplementation, 400 µg daily for 16 weeks in glucose-intolerant Canadian HIV patients, demonstrated an improvement in insulin resistance and a decrease in insulin, triglycerides and upper body and total body fat mass, without any significant effects on glucose, C-peptide or cholesterol indices in the circulation (Aghdassi *et al.*, 2010). Whether this is indicative of underlying chromium insufficiency in HIV or a pharmacological restorative effect cannot be assessed from the study design.

From the aforementioned studies with glucose-intolerant rats (Rhee *et al.*, 2004), we get insights onto immune-related functions of chromium. When the two nutrients, chromium and copper, were both adequate and in a balanced dietary ratio, lymphocyte proliferation was normal. However, in the presence of chromium deficient animal diets, the increased amount of copper actually depressed the lymphocytes function assay. This corroborates earlier findings in hypercholesterolemic postmenopausal women in whom enhanced mitogenic responses of their B- or T-lymphocytes to daily supplementation of 200 µg of chromium had been blunted by a dose of 3.0 mg of copper (Rhee *et al.*, 2002).

27.4.13 Manganese

Manganese is a trace element with a structural role in enzymes, most notably a variety of superoxide dismutase. Its clinical deficiency is restricted to iatrogenic situations, but manganese excess has been reported with increased environmental exposure. The nature of any relationship between manganese and immunity is based on the speculative notions of Kehl-Fie and Skaar (2010) linking the action of the specific zinc transporters to the sequestration of manganese as well. As discussed for zinc above, it is conjectured that there is a beneficial role limiting

access of this metal to pathogens, and hence an additional example of ‘nutritional immunity’ may be involved.

27.5 Multiple nutritional factors

Having reviewed the nutrients, one by one, it behoves this review to consider nutrients in a combined, multiple context. The single nutrient focus is admittedly cumbersome, and deficiencies of more than one nutrient occur in adverse clinical, socio-economic and climatic conditions. As a consequence, a host of investigations have focused on multiple nutrients in field interventions. On the efficacy and safety side, a comprehensive meta-analysis involving pregnant women in 12 developing countries compared the effects of giving a daily multiple micronutrient supplement, containing recommended dietary allowance levels, with standard iron and folic acid supplementation and found no effect on still births or late neonatal mortality (Ronsmans *et al.*, 2009). With a focus on specific infectious disorders, diarrheal disease and respiratory infections, among HIV-positive and -negative Zambian adults, supplementation with 15 micronutrients at around one daily recommended intake had no effect on the incidence of either entity, with a 50% reduction in diarrheal episodes deemed as severe as the only modest effect of the intervention (Kelly *et al.*, 2008).

In terms of multiple micronutrients and immune regulation, the array of micronutrients and dietary constituents characterized as having antioxidant properties have the potential to exhibit a ‘protective role’ in infections caused by bacteria, viruses and parasites, specifically by modulating the inflammatory response (Puertollano *et al.*, 2011). Ravaglia *et al.* (2000) recruited over 50 Italian nonagenarians and centenarians and documented high prevalence of low micronutrient status for multiple biomarkers examined. Focusing on the number and function of NK cells, an important component of innate host defense, the percentage of NK cells was significantly associated only with serum zinc in both sexes and with serum selenium in women only. The latter sex, moreover, showed NK cell cytotoxicity to be positively associated with plasma vitamin E and ubiquinone-10 concentrations. After five months of daily consumption of a dairy product enriched with fish oil, oleic acid, vitamins and minerals or a placebo by Spanish children and adolescents, the most profound effects were in an anti-inflammatory direction, with a decrease in lymphocyte counts and circulating adhesion molecules (Romeo *et al.*, 2011).

27.6 Newer paradigms on the research horizon

27.6.1 Dietary pattern and infection

Food and immunity are firmly linked in the area of allergy in the domain of autoimmunity. The linkage with host defense responses to pathogens and the variance in diets was projected to be an emergent area of inquiry (Solomons, 2007). To some extent, prebiotic and probiotic preparations and bovine colostrum

concentrates can be considered as foods, or at least dietary additives; their respective abilities to reconstitute immune defenses and prevent transmissible diseases have been reviewed (Solomons, 2002; O'Flaherty *et al.*, 2010). The area of interest would be in non-additive constituents of conventional diets.

Many authoritative bodies have provided guidance on general eating patterns that would promote health and prevent disease and disability (WHO/FAO, 2003; WCRF/AICR, 2007; USDA, 2010). They are based on education regarding foods with noxious effects on health and those with constituents that prevent disease. In the context of an intervention trial with oral retinyl palmitate capsules in Sudan, Fawzi *et al.* (1995) noted increased survival in those who consumed higher amounts of provitamin A carotenes in their diet. Either this substance or other compounds in or characteristics of the foods rich in carotenoids could be determinant in the observed association. We have a roster of food components, notable among them polyunsaturated fatty acids and polyphenols, with immunoregulatory roles. A systematic understanding of these and other candidate food constituents and host defenses against infective pathogens is in its infancy.

The flavonoids in fruits and teas regulate genes coding for critical enzymes involved in the inflammatory process; thus it can be speculated that such immunomodulation could be relevant for resistance to infection in humans (González-Gallego *et al.*, 2010), but clinical and epidemiological evidence is lacking. What is available, however, is a well conducted intervention trial with a polyphenol-rich fermented beverage, which was superior to placebo in its ability to increase intracellular glutathione in three immune cell lines, and in NK cell cytotoxicity, in addition to reduced lipid peroxidation and adhesion molecule expression, in healthy volunteers (Schoen *et al.*, 2009). Isoflavones and flavonoid compounds from soy have been shown, in *in vitro* systems, to have potent antiviral effects against an array of RNA and DNA viruses, including adenovirus, herpes simplex virus, human immunodeficiency virus, porcine reproductive and respiratory syndrome virus, and rotavirus (Andres *et al.*, 2009). Channeling these properties for the prevention or treatment of human or veterinary diseases, however, has yet to be realized.

The cell membranes in foods, such as brain, viscera and butter milk, contain a sphingolipid known as gangliosides. These substances influence the microflora and promote immune development in the neonate (Rueda, 2007). These lipids show protozoacidal activity against *Giardia* in *in vitro* systems (Suh *et al.*, 2004). Alcoholic beverages, beer and wine, have antioxidant properties, but only recently have immunity properties of alcoholic beverages been explored. *In vitro*, incubation with beer, actually including alcohol-free beer, produced an anti-inflammatory effect on cultured monocytes through two specific pathways at the metabolic level (Winkler *et al.*, 2006); what this would mean for host defense would depend upon the nature of the pathogen and the stage of the infection. Healthy Spanish volunteers consumed one (women) or two (men) standard rations of 330 mL of beer for 30 days. The absolute circulating counts of immune cells rose in men, but not in women, and the oxidative burst of phagocytosis was enhanced by beer in both sexes (Romeo *et al.*, 2007).

27.6.2 Breastmilk, immunity and nutrition

At the interface of vertical mother-to-child transmission of the HIV virus, it has been shown that the array of innate immune factors in breastmilk can play a role in the infectivity of the virus at the mucosal cell level (Pollakis *et al.*, 2011). One of the features of human milk that cannot be mimicked by breastmilk substitute formulas is its content of immune substances, with their regulatory interaction across the maternal–infant dyad. There is no reason, moreover, to believe that new concepts in host defenses, new pathogen challenges and emerging technology will decelerate in the coming years (Ogra, 2009). New examples of insights include the intact passage of IL-7 from milk into the lymphoid areas of the intestinal wall to modulate development of the baby's T-cell population across the body, as shown in a murine model (Aspinall *et al.*, 2011). Castellote *et al.* (2011) examined the evidence of compensatory adaptation of breastmilk immune constitution with prematurity of infants. Evidence for beneficial adaptation of milk was suggested if the gestational age of the infant was greater than 30 weeks. Milk from mothers of very premature infants below that age had inferior levels for most immune markers as compared to mothers with term deliveries. Finally, with respect to research of this nature, better design and standardization of human milk immune research is called for going forward as suggested by a systematic review by Agarwal and colleagues (2011). Inspecting the literature on an extensive array of immune biomarkers in breastmilk (transforming growth factor (TGF)- β , interferon (IFN)- γ , eotaxin, CCL5, CXCL10, tumor necrosis factor (TNF)- α , monocyte chemoattractant proteins (MCP)-1, IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, sCD14, sIgA, IgG4, IgM), they find wide variation in concentrations and responses. Whether methodological issues, sample size and selection or true biological variation are the source of literature inconsistency requires prompt and authoritative address.

27.6.3 Manipulation of the immune system

Since the identification and synthesis of glucocorticoids, clinical medicine has pursued the external manipulation of the immune system. With recent conceptual breakthroughs and technical interventions, the age of the guided manipulation of the immune system would seem to be poised for acceleration. This, furthermore, may have a nutritional interaction, as seen in the narrative surrounding creating hyperimmune bovine colostrum (Solomons, 2002).

The use of pre- and probiotics represents another nutritional portal of entry for modulating the intestinal milieu to be less hospitable to pathogens, with variable consistency for reducing gastrointestinal risk. The larger concept of the microbiome (Kau *et al.*, 2011), and demonstrations of the potentially protective role of certain patterns of gut microflora, has moved to center stage (Le Huërou-Luron *et al.*, 2010; Fukatsu and Kudsk, 2011). It aligns with the realization of the gut and its associated immunity apparatus, representing the largest and most diverse immune tissue of the body (Satyaraj 2011). Brandtzaeg (2011) sees the gut as the communicator between the environment and the host. A testimony to this emergence, one

of the 2012 Keystone Conference topics is ‘Malnutrition, Gut-Microbial Interactions and Mucosal Immunity to Vaccines’.

The inflammatory response is central at times to the defense against pathogens and, at other times, it can be a determinant of the exhaustion of host metabolic vitality. At a crude level of manipulation, anticytokine antibodies have been introduced in therapy of primarily inflammatory diseases (Nogral and Krueger, 2011), not without serious side effects. Since the cytokine response is governed by a complex signaling chain, the cellular origins and hormonal links can enter the pool of options (De Nitto *et al.*, 2010). Manipulation even includes intervention at the level of epigenetic regulation (Nicodeme *et al.*, 2010). By coincidence, another of the Keystone Conference topics for 2012 is entitled ‘Chemokines and Leukocyte Trafficking in Homeostasis and Inflammation’, highlighting the basic biology that fosters avenues for intervention. How effectively and safely cytokine manipulation will benefit individuals with acute or chronic infections awaits future clinical trials.

27.6.4 Nutritional immunity and host influences on pathogen virulence

Two of the concepts presented are somewhat jarring to scientific sensibilities. They are first the notion of ‘nutritional immunity,’ that is supporting resistance to pathogens by depriving the microbes or parasites of the nutrients they need, even at the transient expense of host’s nutritional wellbeing. This has moved beyond iron to selenium and vitamin E, and most recently to zinc and manganese (Kehl-Fie and Skaar, 2010). Calorie restriction is an established principle in experimental animals to improve immune function. A unique and singular view through the human prism is the relationship of smaller body size in both height and weight to survival from meningococcal meningitis (Perez *et al.*, 2007); as the study comes from Argentina, a careful analysis is whether ‘undernutrition’ is protective or overweight (as in the discussion above) is detrimental.

Respecting the principle of restraint on aggressive nutritional interventions for endemic nutrient deficiencies has begun to temper public health behavior, especially in the aftermath of the Pemba experience (Sazawal *et al.*, 2006); gathering evidence for benign neglect as a control measure for infection, however, runs into bioethical barriers given the reflex assumption of the beneficence of supporting normative nutrition (Solomons, 2010). The conscious induction of nutritional deficiency would be feasible only in a controlled clinical context and only as a means of last resort when pharmacological and surgical options have been exhausted. But nutritional immunity options are likely to be added to the armamentarium of clinical medicine going forward.

The other jarring observation is the emergence of enhanced virulence of pathogens passing through malnourished hosts. This was manifested by the work of Melissa Beck and associates (2004) with the murine Coxsackie virus and its conversion to virulence in passage through selenium-deficient hosts. Louria (2007) cites a similar scenario with influenza virus in mice, and speculates that the porcine N3N2 influenza strain may acquire the capacity for human-to-human transmission by mutations induced by passing through undernourished populations

living in poverty. One wonders if the legion of emerging infectious diseases (Solomons, 2007) has developed virulence on the crest of passage through nutrient-deficient individuals. The paradigm has social implications of how society treats one's neighbor's autonomous right to indulge in any nutrition conduct of their choice (Solomons, 2010). Just as mandatory immunizations have been imposed on reluctant citizens to protect the society as a whole from epidemics, could mandatory nutritional rehabilitation be another enforced obligation to eliminate the situations for virulence transformation of micro-organisms?

27.7 Conclusion

The original, 1959 review by Scrimshaw, Taylor and Gordon (1959) contained over 400 citations. It is likely that primary and review articles relevant to the interaction of malnutrition and nutrition, along with immunity, must have increased by 100-fold by today. This review sought to review and highlight issues emerging within the past decade, but the scope of this failed to allow for some potentially important aspects. It is recognized that essential fatty acids have diverse immune properties, and some of these might involve host defense against infection. Moreover, on the parasitology domain of human and animal pathogens, complex interactions with nutrients and immune mechanisms have come to light, and only a few were touched upon here. Finally, immunization and vaccination are important aspects of host defense, with interactions with nutritional status and concurrent administration of nutrient supplements; this has only been tangentially mentioned in this review.

Scrimshaw, Taylor and Gordon enunciate a paradigm for interactions of nutrition and infection that continues to serve both biological science and public health. The fact that half of all continuing infectious deaths are associated with a low weight-for-age is compelling testimony to the enduring relevance of the paradigm. Half a century of explosive population growth, scientific expansion in immunology and molecular biology, changing population demography, and evolving infectious and nutritional epidemiology places the interaction of nutrition and infection into a myriad of new and relevant contexts.

Source: Solomons (2007).

Box 27.3 Conclusions based on Solomons (2007).

Five years ago, in an update on malnutrition and infection (Solomons, 2007) this author summarized a situation with the words reproduced in Box 27.3. Much of the advancing knowledge published in this short interval runs along the lines of those conclusions. The manner in which genetics and epigenetics can be differential influences in how nutrients act in different individuals, and hence how they support or compromise the immune system, will continue to be revealed, along with the genetic and epigenetic insights to infecting organisms and host defenses, themselves. These are the major prisms through which movement through the brave new world of the newer paradigms on the horizon will be addressed in the next five years.

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FOR REFERENCE PURPOSES ONLY

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Index

- α -casein, 315, 321–2
- α_1 -casein, 325
- α -chemokines, 15
- α -ketoglutarate, 502
- α -lactalbumin, 314
- α -linolenic acid, 468–9
- acute lung injury (ALI), 447
- acute-phase proteins, 11–12
- adaptive immunity, 3–30, 135–7, 632
 - antigen recognition, 19–20
 - B lymphocytes, 24–9
 - characteristics, 633–6
 - interplay of components, 633
 - induction in the gut, 37–44
 - gut-associated lymphoid tissue, 39–40
 - homeostasis-promoting mechanisms, 37–9
 - homing of intestinal immune cells, 40–2
 - intestinal IgA production, 43
 - intestinal immunity during infancy, 43–4
 - level of defense, 6
 - T lymphocytes, 20–4
- adenylate cyclase, 438
- adhesion molecules, 12–19
- adipocytes, 106
 - as immune cells, 110–13
 - cellular interactions in metabolic inflammation, 112
 - TLR4–NF κ B-pro-inflammatory cytokines cascade, 111
- adiponectin, 364
- adipose tissue, 108–9
 - monocytes/macrophages and CLGI, 113–15
- adverse immune reactions
 - classification, 577
 - celiac disease, 594–604
 - foods, 573–606
 - intrinsic and extrinsic host factors, 574
 - IgE-mediated, non-IgE-mediated, and IgE- and non-IgE-mediated food allergy, 576–7
 - IgE-mediated food allergy, 577–88
 - non-IgE-mediated and mixed- or IgE- and non-IgE-mediated food allergy, 588–94
 - adverse reaction to food, 575
 - affinity maturation, 25
 - ageing, 176–8
 - effect on components of immune systems, 636–42
 - cross-sectional studies data, 638–41
 - longitudinal studies data, 641–2
 - overview of effects, 637
 - reasons for observed effects on immunity, 642–5
 - APC alteration leading to altered T-cell activation, 644–5
 - thymic involution, 643–4
 - aglycone naringenin, 381
 - AIDS, 168
 - alanine, 516
 - all-*trans*-retinoic acid, 229–30
 - allergic asthma, 92
 - allergy, 174–6
 - epidemiology, 175
 - probiotics, 275–6
 - evaluation of efficacy, 280–1
 - AMP kinase (AMPK), 503
 - anandamide, 463
 - anaphylaxis, 580–1
 - angiotensin-converting enzymes (ACE)-inhibitory peptides, 315
 - anthocyanidins, 384
 - anthocyanins, 384, 388
 - bioavailability and metabolism, 388
 - structure, classification, and sources, 384
 - antigen presentation, 20
 - antigen presenting cells (APCs), 635
 - antigen recognition, 19–20
 - HLA-independent presentation of antigen, 20
 - antigen–antibody complexes, 11

FOR REFERENCE PURPOSES ONLY

720 Index

- antihistamine, 584
- antioxidant
 - megadoses, 665–8
 - effect of supplementation with
 - placebo, vitamin C, E and β -carotene, 666
 - mechanism that reduces stress-induced or exercise-induced immunodepression, 667
- apigenin, 393
- apoptosis, 509
 - white adipocytes, 113
 - zinc and, 131–2
- arachidonic acid, 467–8
- arachidonoyl ethanolamide (AEA), 462
- 2-arachidonoyl glycerol (2-AG), 462
- arginase, 530
- arginine, 523–38
 - chemical forms and safety of supplementation, 536–7
 - mechanisms by which arginine modulates immune function, 534–6
 - gaseous signalling in immunocytes to affect immune responses, 535
 - mTOR signalling, 535–6
 - nitric oxide, carbon monoxide and hydrogen sulphide, 534–5
 - roles in immune response, 531–4
 - sources in the circulation, 524–8
 - utilisation by multiple pathways, 528–31
 - cells in the immune system, 528–30
 - competition of arginase and NO synthase for arginine in mammalian cells, 529
 - other cell types, 530–1
- α_2 -casein, 321–2
- asthma, 174–6
 - epidemiology, 175
 - mouse models, 175–6
 - selenium status relationship with, 174–5
 - selenium supplementation, 175
 - vitamin D, 256
- asymmetric dimethylarginine (ADMA), 552
- atherosclerosis, 94
- atopy patch testing, 591
- autoimmune disease, 144–5, 277–8
- autoimmunity, 254–5
- autophagy, 510
- avoidance diet, 583
- Avon Longitudinal Study of Parents and Children (ALSPAC), 614
- β -casein, 315, 325
- B cells, 136–7, 179–80
 - development, 24
 - repertoire development, 24–5
- β -chemokines, 15
- β -glucans, 417, 673–4
 - conceptual model for the interaction between β -glucans and innate immunity, 419
 - effects on immunomodulation, 416–29
 - immunological activity, 420–3
 - cooperation of Toll-like receptors and Dectin-1, 422
 - Dectin-1 and innate immunity, 421–2
 - mushroom β -glucans and cellular activation, cytokine and antibody responses, 422–3
 - mushroom β -glucans and receptor activation, 420–1
 - immunomodulation by β -glucans as dietary components, 423–7
 - animal and human studies, 426
 - β -glucan path from diet to gut immune cells, 424–5
 - distribution, metabolism and excretion, 425
 - in vitro* studies, 425–6
 - mushroom β -glucans and preventive health effects, 426–7
 - oral delivery and gastrointestinal absorption, 423–4
 - safety, 427–8
 - structure of different sources, 418
- β -lactoglobulin, 314
- B lymphocytes, 24–9, 635
- bacterial infections, 143–4
- basophil histamine release test, 582
- basophils, 10
- Bifidobacterium bifidum*, 279
- Bifidobacterium infantis*, 274
- Bifidobacterium lactis*, 278
- Bifidobacterium longum*, 273
- bilberry, 365
- bioactive precursors, 315
- body composition, 690–4
- Bowman–Birk inhibitor (BBI), 327
- breastfeeding, 55–7
- breastmilk, 706
- brown adipocytes, 106
- brown adipose tissue (BAT), 109–10
 - characteristics, 109–10
 - differences between WAT, 110
- butyrate, 443

- C-jun N-terminal kinase 1 (JNK), 108
 C-reactive protein (CRP), 360, 465, 624
 caffeine, 664–5
 calcidiol, 246
 cancer, 176–8
 carbohydrates, 297, 662–3
 cardiovascular disease (CVD), 613–15
 caseinomacropptide (CMP), 322, 325–6
 casocidin-I, 331
 catechins, 384
 catechol-O-methyl-transferases (COMT), 385
 CC-chemokine receptor 7 (CCR7), 638–9
 CD3, 624
 CD4, 624, 635
 CD8, 624, 635, 639
 CD28, 639
 CD4 T cells, 252, 272, 598
 CD8 T cells, 252
 CD8 $\alpha\alpha$ T cells, 253
 cell differentiation
 epigenetics regulation of T helper cell, 90–2
 lymphocyte, 271–4
 T helper cells, 84–6
 Th2 and Th1 cells, 230–1
 cell tolerance, 28–9
 cellular communication, 27–8
 ceruloplasmin, 195
 chemokines, 13–15, 15
 chemokine receptor super-family, 14–15
 chromium, 703
 chronic inflammatory diseases, 81–99
 epidemiological trends, 83
 epigenetics, 87–94
 microRNA, 94–9
 T helper cells, 83–7
 chronic low-grade inflammation (CLGI), 105–17
 adipocytes as immune cells, 110–13
 adipose tissue as immune organ, 108–9
 adipose tissue cells impact, 113–16
 involvement in inflammation, 113
 laboratory evaluation, 116–17
 lean adipose tissue anti-inflammatory status, 116
 obesity and development of, 106–8
 white and brown adipose tissue, 109–10
 chronic undernutrition, 691
 chymase, 579
 class I MHC molecules, 19–20
 class II MHC molecules, 19–20
Claviceps purpurea, 575
 ClinOleic, 492, 493
 coeliac disease, 594–604
 coffee, 368–9
 colitis, 172–4
 colony-stimulating factors, 15
 colostrinin (CLN), 324
 colostrum, 676–7
 commensal bacteria, 63–4
 Commission Directive 2003/89/EC, 583
 Commission Directive 2006/142/EC, 583
 common myeloid progenitors *see* myeloid stem cells
 complement activation pathways, 11
 complement receptor 3 (CR3), 420
 concanavalin A (Con A), 485
 conjugated linoleic acids, 466
 copper, 702–3
 CpG islands, 88
 creatine, 553
 critical illness, 172–4
 cyclooxygenase (COX), 462
 cysteine, 548–50
 cytochrome P450, 462
 cytokine storm, 137
 cytokines, 15–19, 439, 585
 sources and effects, 16–19
 daidzein, 384
 Dectin-1, 421
 cooperation of Toll-like receptors and, 422
 innate immunity and, 421–2
 defensins, 301
 deiodinases, 164
 dendritic cells (DC), 424
 Developmental Origins of Health and Disease (DOHaD)
 hypothesis, 612, 613–15
 diabetes type I, 250
 diet, 52–4, 63–4
 dietary fibre, 362–3, 370
 dihomo- γ -linoleic acid (DGLA), 463–4
 divalent metal transporter-1 (DMT1), 195
 DNA demethylation, 89, 93
 DNA methyl transferases (DNMT), 88–9
 DNA modification, 88
 DNMT1, 88–9
 Dutch Hunger Winter of 1944, 614
 Early Nutrition and Immune Development (ENID) Trial, 627
 early nutritional programming, 612–27

- developmental origins of health and disease, 613–15
- infant immune development, 617–23
- infectious disease mortality, 615–17
- long-term immune functions, 623–6
- echinacea, 670–1
- effector cell, 86–7
- effector/memory cells, 42
- eicosapentaenoic acid (EPA), 464
- elemental diets, 593
- elimination diets, 593
- endocannabinoids, 462
- endoprotease B2, 603
- energy-restricted diet, 657
- enterocolitis syndrome, food protein-induced, 590
- enteropathy, food protein-induced, 591
- eosinophilic gastritis, 589–90
- eosinophilic oesophagitis, 589–90
- eosinophils, 9
- epigallocatechingallate (EGCG), 387
- epigenetics, 87–94
 - changes, 92–4
 - mechanism, 88–90
 - regulation, 29
 - regulation of T helper cell differentiation, 90–2
- epitopes, 578
- ergotamine, 522, 575
- eriodictyol, 384
- essential fatty acids, 458
- EuroPrevall, 576
- exercise, 652–78
 - effects on immune system, 653–6
 - factors affecting infection risk in athletes, 654
 - nutrition strategies to limit exercise-induced immune depression, 658–77
 - recommendations, 677
- exercise-induced immune depression
 - nutrition strategies, 658–61
 - nutritional countermeasures, 662–77
 - β -glucans, 673–4
 - caffeine, 664–5
 - carbohydrates, 662–3
 - colostrum, 676–7
 - echinacea and other herbals, 670–1
 - glutamine, 663–4
 - megadoses of antioxidant vitamins, 665–8
 - polyphenols, 671–3
 - probiotics, 674–6
 - vitamin D, 668–9
 - zinc, 669–70
- extracellular signal-related kinase (ERK), 395
- FAHF-2, 585
- farnesyl diphosphate synthase (FPPS), 551
- fermentation, 296–9
- ferritin, 200
- ferroportin-1, 195
- fibromyalgia, 556
- fisetin, 395
- FK506, 535
- flavan-3-ols, 387
- flavanols, 384, 387–8
- flavanones, 381, 384, 387
- flavones, 381, 387
 - bioavailability and metabolism, 387
 - structure, classification and sources, 381
- flavonoids
 - bioavailability and metabolism, 385–8
 - future trends, 406
 - human intervention studies with flavonoids, 397–404
 - immune function, 379–406
 - in vitro* effects on cellular functions, 388–94
 - antigen presenting cells (APC), basophils and mastocytes, 390
 - effects of oxidative burst, 388
 - mechanism of action of immune-modulating activities of flavonoids, 389–90
 - proliferation and cell death, 392–4
 - Th1/Th2 cytokine balance, 390–2
 - molecular mechanisms, 394–7
 - flavonoid modulation of selected transduction pathways involved in immune function, 396
 - structure, classification and sources, 380–4
- flavonols, 380, 386–7
 - bioavailability and metabolism, 386–7
 - structure, classification, and sources, 380
- flavonones, 387
- folate, 697–8
- follicular helper T cells (Tfh), 85
- food allergy, 576
- food bioactive proteins and peptides, 313–33
 - antimicrobial effects, 321–4
 - applications in human health and clinical nutrition, 330–3

- effects on immune function, 324–7
- effects on inflammation, 327–8
- mechanism of action, 328–30
- physiological effects, 317–18
- sources, 314–17
- stability, absorption and site of action, 318–21
- foods
 - adverse immune reactions, 573–606
 - coeliac disease, 594–604
 - IgE-mediated, non-IgE-mediated, and IgE- and non-IgE-mediated food allergy, 576–7
 - IgE-mediated food allergy, 577–88
 - non-IgE-mediated and mixed- or IgE- and non-IgE-mediated food allergy, 588–94
- fruits, 363–7
- G protein-coupled receptors (GPCR), 370, 437
- galacto-oligosaccharides, 295–6, 298
- gangliosides
 - definition and description, 342–3
 - effects on immune function, 349–51
 - immunity, infection and inflammation, 341–54
 - mechanism of action, 346–9
 - sources and intakes, 344–6
- G_{DI}, 347
- G_{D3}, 344, 346, 347, 350, 351
- G_{DI}α, 346, 347
- G_{D1a}, 348, 349
- gelatine, 524
- generally recognised as safe (GRAS), 332–3
- genistein, 384, 390
- Giardia intestinalis*, 691
- gliadin, 597
- glucans, 417
- glutamine, 663–4
 - cell- and immune-modulating properties of, 502–16
 - cell modulation, 507–10
 - chemistry and physiology, 503–4
 - clinical studies, 510–13
 - enteral and oral supply, 513
 - parenteral supply in the ICU, 511–13
 - pharmacokinetics, 511
 - GLN deficiency leads to a state of cellular hibernation, 515
 - immune modulation, 504–7
 - importance of glutamine for lymphocytes, 504–6
 - influence on monocytes and differentiation, 506
 - influence on cell signalling and gene expression, 510
 - starvation effects on aspects of immune cell function, 505
- glutamyl-tRNA synthetase (GLN-RS), 509
- glutathione (GSH), 549
- glutathione peroxidases (Trxrd), 162, 164
- gluten, 598
- gluten-free diet, 601–3
- gluten-specific enteropathy, 594
- glycomacropeptide (GMP), 322
- glycyl-L-glutamine (GLY-GLN), 510
- G_{M1}, 348, 349
- G_{M3}, 344, 349, 350
- GPCR120, 469
- graft *versus* host (GvH) response, 490
- granulocyte macrophage colony-stimulating factor (GM-CSF), 440
- grifolan, 427
- growth, 690–4
- G_{T1b}, 348
- Guidelines for the Diagnosis and Management of Food Allergy, 576
- gut
 - mucosal immunity, 34–65
 - adaptive immunity, 37–44
 - biological variables with impact on developing immunophenotype of infant, 65
 - gut microbial–host interactions, 51–5
 - gut–host communication, 35
 - homeostasis *vs.*
 - hypersensitivity, 57–64
 - IgA and breastfeeding, 55–7
 - secretory immunity, 44–51
 - prebiotics effect, 296–9
 - inulin-type fructans and galacto-oligosaccharides in human studies, 299
- gut-associated lymphoid tissue (GALT), 36, 38–9, 39–40, 268, 406, 424
- compartments, cells and functions of the human mucosal immune system, 40
- gut closure, 47
- gut microbial–host interactions, 51–5
 - diet and microbiota, 52–4
 - epithelium and secretory immunity, 51–2
 - homeostasis tailored by SIgA, 54–5

- haematopoiesis, 642–3
- HEALTHGRAIN, 360
- Heiner syndrome, 588
- heme carrier protein-1 (Hcp1), 195
- heme-oxygenase 1, 195
- hepcidin, 195
- hephaestin, 195
- hespertine, 381
- high-affinity IgE receptors (FcεRI), 578
- histamine, 579
- histone acetyltransferases (HATs0), 437
- histone code, 89–90
- homeostasis, 37–9
 - vs. hypersensitivity in the gut, 57–64
- host *versus* graft (HvG) response, 490
- human aortic endothelial cells (HAECs), 390
- human immunodeficiency virus-1 (HIV-1), 168
- human-milk gangliosides, 344, 347
- human saphenous vein endothelial cells (HSVECs), 488
- humoral immunity, 205–6
- hydroxytyrosol, 496
- hygiene hypothesis, 587
- hyperglycaemia, 370
- hypersensitivity
 - vs. induction homeostasis in the gut, 57–64
- IgA-inducing protein (IGIP), 43
- IgE-mediated food allergy, 577–88
 - diagnosis, 581–3
 - detection of allergen-specific IgE, Plate III
 - oral food challenge, 582–3
 - skin prick test, Plate IV
 - immune mechanism, 578–80
 - management, 583–4
 - presentation, 580–1
 - prevention, 585–8
- IκB kinase beta (IκK-β), 108
- immediate hypersensitivity, 577
- immune function
 - acute effect of exercise, 654–5
 - chronic effect of exercise, 655–6
 - flavonoids, 379–406
 - bioavailability and metabolism, 385–8
 - human intervention studies with flavonoids, 397–404
 - in vitro* effects on cellular functions, 388–94
 - molecular mechanisms, 394–7
 - structure, classification, and sources, 380–4
- nutritional practices of athletes influencing, 656–8
- olive oil, 484–97
 - contribution of non-lipid components to the immunomodulatory effects of olive oil, 495–7
 - effects on various immune functions, 485–92
 - inflammatory disease and olive oil, 493–4
 - olive oil-containing emulsions in clinical nutrition, 492–3
- immune response, 435–48
- immune risk profile (IRP), 641, 642
- immune system
 - ageing and impacts of nutrition, 631–47
 - innate and adaptive, cellular and humoral, 631–6
 - overview of haematopoiesis and thymopoiesis, 636
 - physical and physiological barriers and components of the immune system, 632
- mechanism of action of intestinal bacteria and probiotics, 269–74
- role of prebiotics, 292–308
 - effects, 300–2
- vitamin A, 221–37
 - immunity, 230–6
 - major derivatives and β-carotene, 222
 - metabolism, 223–6
 - retinoids, 228–30
 - sources, forms and dietary recommendations, 222–3
 - transport and metabolism, influence of inflammation, 226–8
- vitamin D, 244–57
 - associated disease, 250–1
 - autoimmunity, 254–5
 - endocrine organ, 254
 - food sources, recommended intakes and epidemiology of deficiency, 248–50
 - immune regulator, 251–4
 - interventions to alter immune function, 257
 - tissue specific effects on immune function, 256–7
- immune tolerance, 576
- immunity, 652–78, 686–709

- effects of exercise on immune system, 653–6
- interaction, 687–90
- nutritional countermeasures to exercise-induced immune depression, 658–77
- research, 704–8
 - immune system manipulation, 706–7
 - nutritional immunity and host influence on pathogen virulence, 707–8
- role of dietary selenium, 157–82
 - against parasites, 170–2
 - allergies and asthma, 174–6
 - bacterial and fungal infections, 168–70
 - cancer and ageing, 176–8
 - continuum of selenium status, 158
 - critical illness, sepsis and colitis, 172–4
 - effect of decreased intake on selenoprotein synthesis, 165–6
 - mechanism, 178–80
 - nutrition, 158–62
 - selenoprotein, 162–5
 - viral immunity, 166–8
- role of iron, 193–213
 - impaired mechanism in iron deficiency, 209–11
 - infection, 211–13
 - iron deficiency, 201–7
 - iron overload, 207–9
 - overview, 193–201
- role of zinc, 123–46
 - effects on immune function, 132–7
 - human health and clinical nutrition, 138–45
 - molecular mechanism of action, 128–32
 - nutritional background, 124–8
 - selected micronutrients, 694–704
- immunoglobulin A (IgA), 35, 56
 - functions in gut defence, 49–51
 - immune control, 55–7
 - intestinal production, 43
 - subclass distribution and J-chain expression of IgA-producing plasma cells, 44
- immunological synapse, 640
- immunomodulation, 416–29
- immunonutrition, 538
- immunoregulation, 28–9
- immunosenescence, 636, 637–8
- immunotherapy, 584
- inducible nitric oxide synthase (iNOS), 389, 528
- infection, 686–709
 - growth and body composition, 690–4
 - human immune regulation and energy metabolism evolution, 693
 - obesity and overweight conditions, 692–4
 - stature-deficit conditions, 691–2
 - weight-deficit conditions, 690–1
 - interaction, 687–90
- iron, 211–13
- probiotics, 275
 - dietary pattern, 704–5
 - selected micronutrients, 694–704
 - vitamin D, 255–6
- inflammation
 - modulation by short-chain fatty acids, 435–48
 - role of dietary selenium, 157–82
 - allergies and asthma, 174–6
 - bacterial and fungal infections, 168–70
 - cancer and ageing, 176–8
 - continuum of selenium status, 158
 - critical illness, sepsis and colitis, 172–4
 - effect of decreased intake on selenoprotein synthesis, 165–6
 - immunity against parasites, 170–2
 - mechanism, 178–80
 - nutrition, 158–62
 - selenoprotein, 162–5
 - viral immunity, 166–8
 - role of iron, 193–213
 - role of prebiotics, 292–308
 - role of zinc, 123–46
- inflammatory bowel disease (IBD), 276–7, 327–8, 443–6
 - olive oil and, 493–4
- inflammatory processes
 - long-chain fatty acids, 457–75
 - effects of specific fatty acids on inflammation, 465–73
 - fatty acid composition importance for function of involved cells, 461–2
 - lipid mediators produced from n-6 and n-3 fatty acids, 462–5
 - plant foods and, 359–71
 - coffee and tea, 368–9
 - dietary fibre, 362–3
 - mechanisms contributing to the anti-

- inflammatory effects of plant foods, 369–71
 - nuts, 368
 - soy, 367
 - vegetables and fruits, 363–7
 - whole grain and refined grain, 360–2
- influenza virus, 166–7
- innate immune recognition, 4–7
 - pattern-associated molecular patterns, 6
 - TLR trafficking and signalling, 5
- innate immune response *see* innate immunity
- innate immunity, 3–30, 134–5, 632
 - adhesion molecules and signalling, 12–19
 - leukocyte–endothelial cell interactions, 12
 - cellular components, 7–10
 - haematopoietic stem cell-derived lineages, 8
 - characteristics, 633–6
 - innate immune recognition, 4–7
 - level of defence, 6
 - soluble factors in innate defence, 10–12
- insulin receptor substrate 1 (IRS-1), 108
- insulin resistance, 106–108
- intercellular adhesion molecule (ICAM)-1, 488
- interferon gamma (IFN- γ), 528
- interferon-stimulated genes (ISG), 555
- interferons, 15
- interleukin (IL)-2, 578
- interleukin (IL)-4, 528, 578
- interleukin (IL)-5, 578
- interleukin (IL)-6, 360, 465
- interleukin (IL)-10, 301, 302
- interleukin (IL)-13, 278
- interleukins, 15
- intestinal bacteria, 267–83
 - mechanism of action on the immune system, 269–74
 - CD4 T cell subsets, 272
 - human pattern-recognition receptors and their ligands, 270
 - lymphocyte differentiation, 271–4
 - microbial signals recognition by epithelial and innate immune cells, 269–71
- intestinal immune cells
 - induction and homing, 40–2
- intestinal inflammation, 302–5
- intestinal parasites, 172
- intestinal tolerance, 58–9
- intracellular zinc, 128–30
- intraepithelial lymphocytes (IEL), 598
- intrauterine growth retardation (IUGR), 615, 621
- inulin, 304
- inulin-type fructans, 295, 298, 305, 307–8
- iron, 699–701
 - deficiency, 201–7
 - immunity and inflammation, 193–213
 - impaired immunity mechanism in iron deficiency, 209–11
 - infection, 211–13
 - overload, 207–9
 - overview, 193–201
 - absorption and transport proteins, 195–200
 - assessment in the human body, 194–5
 - proteins involved in iron homeostasis, 196–9
 - proteins role with homeostasis in immunity, 200–1
- iron deficiency, 201–7
 - cell function, 201–3
 - effect of natural killer (NK) cell activity, 206–7
 - effect on humoral immunity, 205–6
 - effect on macrophage functions, 206
 - effect on neutrophil functions, 206
 - effect on pro- and anti-inflammatory cytokines, 203–4
 - impaired immunity mechanism, 209–11
- iron overload, 207–9
 - lymphocyte functions, 207–8
 - macrophage functions, 208
 - natural killer (NK) cell functions, 208–9
 - neutrophils, 208
- iron regulatory element protein-2 (IREP2), 195
- irritable bowel syndrome (IBS), 305
- IRT5, 273
- ischaemia tissue injury, 448
- ischaemic heart disease, 514
- isoflavones, 384, 387
- isotype switching, 25
- isracidin, 329
- Ivelip, 493
- J-shaped model, 653
- jun N-terminal kinase (JNK), 395
- jun nuclear kinase (JNK), 509
- κ -casein, 315, 321–2, 325
- kaempferol, 380, 391, 392

- kwashiorkor, 691
- L-2-oxothiazolidine-4-carboxylate (OTZ), 554
- L-alanyl-L-glutamine (ALA-GLN), 510
- L-citrulline, 537
- lactase phloridizin hydrolase (LPH), 385
- Lactobacillus*, 274, 278, 279, 280, 282
- lactoferricin, 329
- lactoferrin, 323, 326–7, 329
- lactose intolerance, 575
- lactosylceramide, 421
- laminarin, 423–4
- lean adipose tissue, 116
- Learn Early About Peanut allergy (LEAP study), 586
- lectin, 11
- lentinan, 418, 426
- Lentinus edodes*, 426
- leucine-rich repeat (LRR), 420
- leukocyte common antigen (LCA), 638
- leukocyte 5-lipoxygenase, 496
- leukotrienes (LT), 461
- linoleic acid, 466–7
- lipid-bound sialic acid (LBSA), 342, 344, 346
- lipoxins, 462
- lipoxygenase (LOX), 462
- listeriosis, 169
- long-chain fatty acids, 457–75
 - effects of specific fatty acids on
 - inflammation, 465–73
 - α -linolenic acid, 468–9
 - arachidonic acid, 467–8
 - conjugated linoleic acids, 466
 - hierarchy of effects of fatty acids on
 - inflammation, 473
 - linoleic acid, 466–7
 - marine n-3 fatty acids, 469–72
 - qualitative depiction of relative
 - effects fatty acid on
 - inflammation, 473
 - saturated fatty acids, 465
 - trans fatty acids, 466
 - fatty acid composition importance for
 - function of involved cells, 461–2
 - lipid mediators produced from n-6 and n-3 fatty acids, 462–5
 - nomenclature and common dietary
 - sources, 459–60
 - pathway biosynthesis of fatty acids, 458
- low-grade inflammatory state, 694
- luteolin, 390, 393
- Lyc-O-Mato, 366
- lymphocyte, 271–4, 442
- lymphocyte function-associated antigen-1 (LFA-1), 488
- lymphoid tissue, 27–8
- M cells, 424
- M1 and M2 macrophages, 114
- macrophage-stimulating factor (M-CSF), 440
- macrophages, 9, 113–15
- macrophages/monocytes, 440–2
- magnesium, 699
- maintenance methyl transferase, 88
- major histocompatibility complex (MHC), 635
- major histocompatibility I
 - molecules, 19–20
- malnutrition, 686–709
 - interaction with infection and
 - immunity, 687–90
 - multiple nutritional factors, 704
 - research, 704–8
 - selected micronutrients, 694–704
- mammalian (or mechanistic) target of rapamycin (mTOR), 524
- manganese, 703–4
- marine n-3 fatty acids, 469–72
- mast cells, 10, 578
- Maternal and Infant Nutrition
 - Interventions, Matlab (MINIMat) trial, 620
- Matlab Health and Demographic Surveillance System, 619
- measles virus, 236
- mechanism of action
 - food bioactive proteins, 328–30
 - gangliosides, 346–9
 - intestinal bacteria, 269–74
- metabolic syndrome, 305–6
- methionine, 548
- methionine adenosyl transferase (MAT), 558
- microbial-associated molecular patterns (MAMPs), 7, 36
- microbial cell walls, 11
- microbiota, 52–4, 435
- microflora, 435
- microglial cells, 441
- micronutrients, 694–704
- microRNA, 29, 94–9
- milk, 315
- miR17–92, 97
- miR-155, 96, 98

- miR-181a, 97–8
- mitogen-activated protein kinase (MAPK), 108, 421, 524
- mixed lymphocyte culture (MLC), 392
- mixed mediated food allergy, 588–94
- model situation, 83
- monocyte chemoattractant protein (MCP)-1, 365
- monocytes, 9, 113–15
- monounsaturated fatty acid (MUFA), 486
- Montelukast, 594
- mouse B cell hybridoma cells, 422
- mucosa-associated lymphoid tissue (MALT), 39
- mucosal immunity
 - biological variables with impact on developing immunophenotype of infant, 65
 - gut, 34–65
 - gut adaptive immunity, 37–44
 - gut microbial–host interactions, 51–5
 - gut–host communication, 35
 - IgA and breastfeeding, 55–7
 - induction homeostasis *vs.*
 - hypersensitivity in the gut, 57–64
 - commensal bacteria and diet, 63–4
 - decision-making in the mucosal immune system, 60
 - genes *vs.* the environment, 62–3
 - sensing microbial components, 59–61
 - secretory immunity, 44–51
- multiple sclerosis, 250
- multisystem organ failure (MSOF), 557
- myeloid stem cells, 7
- n-3 and n-6 PUFA, 659
- N-acetylcysteine (NAC), 554
- N-acetylglutamate (NAG) synthase, 527
- National Health and Nutrition Examination Survey (NHANES III), 360
- natural killer cells, 10
- natural resistance antimacrophage protein-2 (NRAMP2), 195
- necrotising enterocolitis (NEC), 351
- neuroprotectins, 464
- neutrophils, 8–9, 439–40
- NFAT (nuclear factor of activated T-cells), 421–2
- NFκB, 394
- NK receptor-mediated cytotoxicity, 596
- NOD1 and NOD2, 271
- non-IgE-mediated food allergy, 588–94
 - diagnosis, 591–2
 - immune mechanisms, 588–9
 - management, 593–4
 - presentation, 589–91
- non-tropical sprue, 594
- Nrf2, 394
- nuclear factor kappa B (NFκB), 465
- nutrigenomics, 117
- nutrition, 652–78
 - countermeasures to exercise-induced immune depression, 662–77
 - practices of athletes influencing immune functions, 656–8
 - prebiotics, 306–8
 - recommendations, 677
 - selenium, 158–62
 - strategies to limit exercise-induced immune depression, 658–61
 - zinc, 124–8, 138–45
- nutritional immunity, 316, 707–8
- obesity, 106–8, 692–4
 - white adipocytes apoptosis, 113
- 1,25(OH)₂-vitamin D, 246–7, 252–3, 257
- 25(OH)-vitamin D, 246
 - normal range and measurement, 249–50
- oleuropein, 496
- oligofructose, 304
- oligofructose-enriched inulin, 298
- oligofructose-enriched lactulose, 298
- olive oil, 484–97
 - containing emulsions in clinical nutrition, 492–3
 - contribution of non-lipid components to the immunomodulatory effects of olive oil, 495–7
 - high-oleic sunflower oil diet on rat spleen lymphocyte natural killer cell activity, 495
 - effects on various immune functions, 485–92
 - host defence and, 491–2
 - inflammatory disease and, 493–4
- oral allergy syndrome, 581
- oral food challenge, 582–3
- oral tolerance, 36
 - in humans, 57–8
- osteomalacia, 250
- overweight, 692–4
- oxoproline, 554
- p38 kinase, 395
- paracetamol, 554–5
- parasitic infections, 144

- parathyroid hormone, 247–8
- pathogen-associated molecular pattern (PAMP), 4, 7, 420, 632
- pathogen virulence, 707–8
- pattern-recognition receptors (PRRs), 4–7, 36, 269, 420
- peanut allergy, 586
- peripheral blood mononuclear cells (PBMNC), 425, 486
- peroxisome proliferator-activated receptor γ (PPAR γ), 507
- peroxynitrite, 533
- phaseolamin, 316
- Phenol-Explorer, 380
- phenylketonuria, 575
- phorbol myristate acetate (PMA), 392
- phospholipase C, 438
- phytochemicals, 370
- phytohaemagglutinin (PHA), 392
- plant foods, 359
 - coffee and tea, 368–9
 - dietary fibre, 362–3
 - inflammatory processes and, 359–71
 - mechanisms contributing to the anti-inflammatory effects, 369–71
 - nuts, 368
 - soy, 367
 - vegetables and fruits, 363–7
 - whole grain and refined grain, 360–2
- plasma Tmax, 386
- plasminogen activator inhibitor-1 (PAI-1), 360
- poliovirus, 167
- polymers of IgA (PIgA), 44
- polyphenols, 671–3
- polysaccharide conjugate vaccine, 626
- polyunsaturated fatty acids (PUFAs), 367, 486, 659
- ponderal index, 614
- pouchitis, 304
- pre-vitamin D₃, 244–5
- prebiotics
 - description of products with established prebiotic effect, 294
 - effect in the gut, 296–9
 - inulin-type fructans and galacto-oligosaccharides in human studies, 299
 - human health and clinical nutrition, 306–8
 - immune system and inflammation, 292–308
 - intestinal inflammation and metabolic syndrome, 302–6
 - lymphoid follicles for induction and regulation of immune responses, 300
 - natural sources and products, 294–6
 - inulin-type fructans content of miscellaneous plants, 296
- PREDIMED, 494
- premonocytic U937, 509
- pro-inflammatory cytokines, 634
- proanthocyanidins, 384
- probiotics, 267–83, 674–6
 - efficacy in animal models, 274–8
 - efficacy in humans, 278–83
 - mechanism of action on the immune system, 269–74
- procyanidins, 384
- productin of cytokines, 442
- programming infant immune development, 613, 617–23
- programming infectious disease mortality, 613, 615–17
 - Kaplan–Meier survival plots by season of birth, 616
- programming long-term immune functions, 613, 623–6
- proline-5-carboxylate (P5C) synthase, 527
- proline oxidase, 527
- prolyl endopeptidases (PEP), 603
- prostaglandins (PG), 461
- proteases, 579
- protectins, 464
- Protein Digestibility-Corrected Amino Acid Score (PDCAAS), 546
- protein–energy malnutrition (PEM), 690
- protocolitis, food protein-induced (allergic), 590
- pulse lectins, 330
- quercetin, 380, 390, 391, 393, 395
- rafts, 461
- reactive oxygen species (ROS), 388, 394, 549
- refined grain, 360–2
- regulatory T (Treg) cells, 85, 91
- remodelling hypothesis, 637
- resolvins, 464
- respiratory burst, 8
- retinoic acid (RA), 221–2
 - lymphocyte homing to the gut, 234–5
 - mucosal T cell activation, 234
 - sources, 235–6
 - T-regulatory and Th17 lineage development, 232–3

- differentiation of a naïve CD4 T cell, 232
- Th2 and restrain Th1 cell differentiation, 230–1
- retinoids, 223
 - inhibit measles virus replication, 236
 - metabolism, 228
 - pro-inflammatory and anti-inflammatory, 228–30
 - retinol metabolism in skin, 229–30
 - skin health, 230
 - treatment, 229
 - tissue, 225
- retinol *see* Vitamin A
- rickets, 250
- rutin, 395
- S-adenosylhomocysteine (SAH), 548
- S-adenosylmethionine
 - metabolism, 550–3
- S-adenosylmethionine decarboxylase (SDC), 548
- S-adenosylmethionine (SAM), 548
- Salmonella typhi* Vi vaccine, 624–5
- salmonellosis, 169
- sarcosine, 553
- saturated fatty acid, 465, 489
- schizophyllan, 419, 427
- scleroglucan, 423–4
- sE-selectin, 468
- secretory IgA (SIgA), 34, 35–6, 45–6, 47, 48, 54–6
- secretory IgM (SIgM), 34, 45–6, 47, 48, 55–6
- secretory immunity, 44–51, 51–2
 - gut epithelial barrier, 47–9
 - IgA functions in gut defence, 49–51
 - secretory antibodies production, 44–7
- selenium, 702
 - allergies and asthma, 174–6
 - bacterial and fungal infections, 168–70
 - cancer and ageing, 176–8
 - continuum of selenium status, 158
 - critical illness, sepsis and colitis, 172–4
 - effect of decreased intake on
 - selenoprotein synthesis, 165–6
 - immunity against parasites, 170–2
 - inflammation and immunity, 157–82
 - mechanism, 178–80
 - nutrition, 158–62
 - deficiency and toxicity, 158–9
 - geography and status, 159
 - metabolism and selenoprotein synthesis, 159–62
 - selenoprotein, 162–5
 - viral immunity, 166–8
- selenoprotein, 162–5
 - synthesis, 159–62
- sepsis, 172–4, 446–8, 557
- shiitake, 426
- short-chain fatty acids (SCFAs), 370
 - effect on inflammation, 443–8
 - effect on leukocyte function, 438–42
 - effect on macrophage production of inflammatory mediators, 441
 - inflammatory and immune responses modulation, 435–48
 - mechanisms of action, 437–8
 - schematic overview of the signaling pathways, Plate II
 - perspectives of SCFAs and inflammation, 448
 - physiology, 435–7
 - schematic representation of the production, absorption and handling, Plate I
- signal-joint T cell receptor-rearrangement excision circle (sjTRECE), 621–3
- distribution of concentration in T cells, 622
- signal transducer and activator of transcription (STAT1), 555
- signalling molecules, 12–19
 - structure and ligands, 13
- signalosomes, 640
- skin prick test, 581
- soluble factors, 10–12
- soluble intercellular adhesion molecule-1 (sICAM-1), 467
- soluble tumour necrosis factor (TNF)- α receptor 2 (sTNFr2), 360
- soluble vascular cell adhesion molecule-1 (sVCAM-1), 467
- soy, 367
- soybean oligosaccharides, 296
- soymetide, 327
- sphingolipids, 344
- St Anthony's fire, 575
- Staphylococcus aureus* enterotoxin B (SEB), 391
- sulfo-transferases (SULT), 385
- sulphur amino acids, 544–60
 - cysteine synthesis and metabolism, 548–50
 - effect on illnesses, 556–60
 - metabolism – transmethylation and trans-sulphuration, 544
 - methionine metabolism, 548

- paracetamol overdose as paradigm for supplementation, 554–5
- requirements following injury of injection, 553
- S-adenosylmethionine and infection, 555–6
- S-adenosylmethionine as portal for polyamine synthesis, 547
- S-adenosylmethionine metabolism, 550–3
 - free radicals, 551
 - methylation reactions, 551–3
 - polyamines, 550–1
- structure, 545
- supplementation with methionine and cysteine, 553–4
- suppressor of cytokine signalling, 108
- Syk, 422
- systemic inflammatory response syndrome (SIRS), 558
- systemic lupus erythematosus (SLE), 93

- T cell-antigen receptor complex, 21–2
- T cell-dependent B cell responses, 25–6
- T cell-independent B cell responses, 25–6
- T cell proliferation, 442
- T cell receptor (TCR), 635, 640
- T cells, 135–6, 179
 - CLGI, 115–16
 - development, 20–1
 - function regulation by MiRNA, 96–9
 - microRNAs in T cell biology, 97
 - subpopulations, 22–3
- T helper cells, 23–4, 83–7, 578
 - differentiation, 84–6
 - epigenetics regulation, 90–2
 - flexibility and plasticity of effector cell, 86–7
 - T cell differentiation and plasticity, 84
- T lymphocytes, 19–20, 20–4, 635
- T regulatory cell, 232–3
- tangeretin, 390, 393
- tea, 368–9
- Th1 cells, 84, 90–1, 92, 230–1, 254–5, 271
- Th2 cells, 84–5, 90–1, 92, 98, 230–1, 271
- Th17 cells, 85, 232–3, 255, 271–2
- Th22 cells, 85
- thioprolone, 554
- thioredoxin reductases, 164
- thrifty gene, 692
- thymic epithelial cell (TEC), 644
- thymic index, 618, 620
- thymus, 617
 - relative size variation with time of year, 620
- thyroglobulin, 623
- thyroid peroxidase, 623
- tissue hypoxia, 106
- tissue transglutaminase (TTG), 597
- Toll-like receptors (TLRs), 7, 269, 420
- TRAIL (TNF-related apoptosis-inducing ligand), 509
- trans fatty acids, 466
- transferrin, 200, 315
- transforming growth factor (TGF)- β , 328, 330
- tretinoin (Retin-A), 229
- trypanosomes, 171
- tryptase, 579
- tuberculosis, 169–70, 615
- tumour necrosis factor- α (TNF- α), 421, 488, 528
- type I hypersensitivity, 577

- ultrasound, 617
- uridine-50-diphosphate glucuronosyl-transferases (UGTs), 385

- vaccination, 145
- vascular cell adhesion molecule-1 (VCAM-1), 488
- vegetables, 363–7
- viperin, 551
- viral immunity, 166–8
- viral infections, 140, 143
- vitamin A, 694–5
 - immune system, 221–37
 - immunity, 230–6
 - polarised cell phenotypes, 231–2
 - Th2 and restrain Th1 cell differentiation, 230–1
 - major derivatives and β -carotene, 222
- metabolism, 223–6
 - enzymatic processing and nuclear functions, 225–6
 - major processes, 224
 - tissue retinoids, 225
 - uptake and distribution, 223–5
- retinoids, 228–30
- sources, forms and dietary recommendations, 222–3
 - nutritional forms, 222
 - pharmacological forms, 223
 - recommended intakes, 222–3
- transport and metabolism, influence of inflammation, 226–8

FOR REFERENCE PURPOSES ONLY

732 Index

- vitamin A deficiency, 223
- vitamin B12, 698–9
- vitamin C, 697
- vitamin D, 588, 668–9, 695–6
 - action, 246–7
 - active form biosynthesis, 244–6
 - associated disease, 250–1
 - asthma, 256
 - autoimmunity, 254–5
 - control by PTH, 247–8
 - endocrine organ, 254
 - food sources, recommended intakes, 248–50
 - immune regulator, 251–4
 - role in CD4+ T cell development and function, 253
 - immune system, 244–57
 - infection, 255–6
- vitamin D₃, 245
- vitamin D deficiency, 248–9
- vitamin E, 697
- whey proteins, 322–3, 326
- white adipocytes, 106
 - large trunkal and CLGI, 112
 - obesity and apoptosis, 113
- white adipose tissue (WAT), 109–10
- whole grain, 360–2
- yeast infections, 170
- zinc, 669–70, 701–2
 - effects on immune function, 132–7
 - human health and clinical nutrition, 138–45
 - bioavailability of zinc salts, 140
 - effects on various disease, 140–5, 141–2
 - impact on inflammatory process, 137–8
 - molecular mechanism of action, 128–32
 - apoptosis, 131–2
 - intracellular, 128–30
 - signalling, 130–1
 - nutritional background, 124–8
 - assessment, 125–6
 - content in food, 127
 - recommended intake, 126–8
 - uptake and risk factors for deficiency, 127
 - role in immunity and inflammation, 123–46
- zinc deficiency, 125, 126
 - effects in elderly people, 133–4
- zinc signalling, 130–1
- zinc wave, 129
- zonulin, 604
- zymosan, 422