Nutrition and Immunity

Maryam Mahmoudi
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Editors

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We wish to dedicate this book to our daughters, Ariana and Arnika, with the hope that progress in science may result in better quality of life for the next generations, and at the same time that international collaboration in research will happen without barriers.

Whatever we have learnt, comes from our mentors. This book is therefore dedicated also to all of them, but most importantly to the patients and their families whose continuous support has guided us during the years.

Maryam Mahmoudi, MD, PhD
Nima Rezaei, MD, PhD

Preface

There is a commonsense link between nutrition and health – eating too little correlates to infection and immunodeficiency disorders while eating too much to obesity and other metabolic disorders. But that is actually what we want to know is where does this link arise from? As outlined in the first Chapter, with varieties of cells, organs, and mechanisms, the immune system has the ability to inspire the whole body. Indeed, it is our body's sixth sense!

Like other cells, immune cells use energy in order to accomplish their mission of helping to maintain the body's homeostasis. Supporting this, undernutrition and immunodeficiency are strongly blend together. Studies investigating the effects of deficiency of a single nutrient have concluded that there are nutrients essential to the proper functioning of the immune system. Among which are vitamin A, group B vitamins, vitamin C, vitamin D, vitamin E, vitamin K, zinc, and selenium. Chapters 2, 3, 4, 5, 6, 7, 8, and 9 provide an overview of the roles that these micronutrients play in immune homeostasis. In contrast, overnutrition is accompanied by chronic nutrient-energy stress. Thereby, the multiple waves of inflammation are spread throughout the body, including the adipose tissue, brain, liver, lung airways, and pancreatic islets. Such widespread inflammatory processes, collectively called metaflammation, contribute to different immunometabolic diseases, including diabetes, cardiovascular diseases, allergy, asthma, neurodegenerative diseases, and cancers. Chapters 13, 14, 15, 16, 17, 18, 19, 20, and 21 discuss the contribution of nutrition to inflammation in a disease-specific manner.

Of particular importance to the present Book is the gut that with the largest population of microflora plays an essential role in the regulation of the balance between immune tolerance and inflammation. Interestingly, the composition of the gut microbiota is under the direct influence of what we eat. Chapter 10 describes how dietary factors through modifying the gut microbiota would alter immune responses. Then, the immune-modulatory effects of flavonoids and glucan in particular are discussed in Chaps. 22 and 23, respectively.

After giving the reader the feeling that the contribution of nutrition to immunity is as obvious as a bright day, the Book is finished by Chaps. 24 and 25 pointing out that the effect of nutrition on immunity is not superficial, but runs deep to the genome and epigenome, and therefore is not temporary, but can be transferred to other lineages.

With the above-described outline, it will become really tangible for the readers of the Book *Nutrition and Immunity* that healthy dietary patterns that

are within reach can provide immunity against disorders that damage the brain, gut, and other body areas and affect ourselves and our children. In this manner, immunonuritional programming offers the most readily available strategy for saving the world people's health.

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Introduction

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Key Points

- Because of its ability to produce pleiotropic effects and influence whole-body physiology, the immune system is considered the health's sixth sense.
- The immune system is trainable, and heritable and nonheritable factors closely interact to shape the immune health.
- The gut is a direct route to the assembly of the immune system by dietary factors.
- Nutrition and foods directly affect the composition of the gut microbiota, which, in turn, would influence the immune homeostasis.
- Undernutrition and immunodeficiency are strongly blended together, while overnutrition is accompanied by chronic widespread inflammation.
- This chapter is an introduction to the book *Nutrition and Immunity*.

The Immune System: The Health's Sixth Sense

The Immune System

Immune Cells

The pluripotent hematopoietic stem cells resident in the bone marrow either directly or indirectly produce all types of immune cells [1]. The division of a pluripotent hematopoietic stem cell results in two progenitor cells: common lymphoid progenitor and common myeloid progenitor. The former, in turn, gives rise to B cells and T cells, which then respectively would differentiate into effector plasma cell and activated T cell. The latter generates two progenitor cells at later stages: granulocyte/macrophage progenitor and megakaryocyte/erythrocyte progenitor. Circulating granulocytes (also known as polymorphonuclear leukocytes) including neutrophils, eosinophils, basophils, unknown precursors, monocytes, and immature dendritic cells (DCs) come from granulocyte/macrophage progenitor. Once they migrate into tissues, monocytes, in turn, differentiate into mast cells, macrophages, and immature DCs. Immature DCs mature in lymph nodes. Megakaryocyte/erythrocyte progenitor turns into megakaryocyte and erythroblast lineages, which respectively lead to the generation of platelets and erythrocytes.

Immune Organs

Lymphocyte differentiation occurs in central (or primary) lymphoid organs that are bone marrow (B cells) and thymus (T cells) [1]. The differentiated lymphocytes enter into the bloodstream and then migrate to the peripheral (or secondary) lymphoid organs including the lymph nodes, spleen, and mucosa-associated lymphoid tissues (appendix, Peyer's patches, and gut-associated tonsils), where they are activated in an antigen-dependent manner to become effector cells. Lymphatics are filled with the lymph fluid, which is required for transport of antigens to lymph nodes and for recirculation of activated lymphocytes to the blood.

Immune Mechanisms

When pathogens invade the body, the innate immunity first elicits responses in a rapid and nonspecific manner, and then the adaptive immunity provides responses that are slower but more specific for the pathogen. The observation in different species (plants, fruit flies, and mammals) of similar mechanisms that underlie innate immune recognition provides evidence that the innate immune mechanisms are highly conserved throughout evolution, while the adaptive immunity is restricted to vertebrates. The innate immunity is, thus, known as the ancient gatekeeper, whereas the adaptive immunity is a relatively modern immunity.

Innate Immunity

The innate immune system can discriminate self from nonself yet is unable to make specific responses against pathogens. Pattern recognition receptors (PRRs) including toll-like receptors (TLRs) are germline-encoded receptors that systematically interact with pathogen-associated molecular patterns (PAMPs), thereby making it possible to discriminate between self and nonself [2]. Microbes share the same PAMPs, for example, lipopolysaccharide (LPS) in gram-negative bacteria and peptidoglycan (PG) in gram-positive

bacteria (for review, see [2]). Once infectious nonself agents are recognized by PRRs, polymorphonuclear leukocytes along with mast cells, macrophages, and natural killer (NK) cells develop into effector cells that contribute to the initiation of the inflammatory response [3].

Adaptive Immunity

When the innate immunity cannot effectively resolve infection, it says the adaptive immunity to continue efforts to distinguish self and nonself. More precisely, the innate immunity employs antigen-presenting cells, in particular DCs, which express costimulatory molecules CD80 and CD86 to introduce the infectious agent to the adaptive immunity. Unlike the innate immunity, which uses receptors that are fixed in the genome, the gene rearrangement is required for the generation of the adaptive immune receptors. As a result, the adaptive immune system is armed with a full repertoire of antigen receptors built up from random gene segment rearrangements [4]. With the distribution of these receptors over the effector cells, i.e., antibody-producing cells and activated T cells, the adaptive immunity can specifically recognize pathogens and related proteins, carbohydrates, lipids, and nucleic acids [3] whereby the immunological memory is made.

Immune Disorders

With the abovementioned cells, organs, and mechanisms, the immune system can have pleiotropic effects that are truly tangible throughout the body. Therefore, we can expect that the human health is influenced by the immune system in many ways. Disorders of the immune system cover a full spectrum from immunodeficiency disorders to overactive immune system and autoimmune disorders. Overall, recurrent infections are the most common manifestation of immunodeficiency. This implicates that the innate immune responses are collectively more impaired than the adaptive immune responses in immunodeficiency. However, both the innate and adaptive immunities can be affected in both inherited (primary) and acquired immunodeficiency syndromes (AIDS). In contrast, an overactive immune system as seen in inflammatory diseases and allergies and autoimmune disorders predominantly results from the unwanted activity of the adaptive immunity. Also, an imbalanced immune homeostasis has been associated with a myriad of medical conditions such as cancer, neurological diseases, and psychiatric disorders [5–7].

The Immune System Is Trainable

Previously, it was a privilege of the adaptive immunity rather than the innate immunity to make immunological memory. Recent studies have provided evidence that the innate immunity as well as the adaptive immunity can be trained such that it can become resistant to reinfections [8, 9]. Now that the possibility of training the overall immunity has been sublated into literature, there is pressure to explore factors affecting the immune system, more than before. It is known that heritable and nonheritable factors interact to determine the immune health. Of note, study of healthy twins pointed to the substantial share that nonheritable factors, in particular nutritional factors, pathogens, and vaccinations, have in establishing immunological parameters involving cell phenotype frequencies, serum protein concentrations, and cell signaling responses to cytokine stimulation [10]. Of particular interest to the present chapter is how nutrition may have on the immune system.

Nutrition: A Direct Route to the Assembly of the Immune System

Energy Balance Is the Key to Immune Homeostasis

Inevitably, all cell types including immune cells use energy in order to accomplish their mission of helping to maintain homeostasis. Lack of adequate energy supplies and nutrients is, therefore, one important reason for the poor quality of immune programming [11]. Supporting this, undernutrition and immunodeficiency are strongly blended together [12]. Immunodeficiency, in turn, would increase the risk of infections.

In contrast, overnutrition is accompanied by chronic nutrient-energy stress. Thereby, the multiple waves of inflammation are spread throughout the body, including the adipose tissue, brain, liver, lung airways, and pancreatic islets. Such widespread inflammatory processes – which are collectively called metaflammation – contribute to different immunometabolic diseases including diabetes, cardiovascular diseases, asthma, neurodegenerative diseases, and cancers.

In this manner, clearly, the immune homeostasis remains healthy as long as energy balance is successful.

Nutrition and Immunity Are Evolutionary Interrelated

The Evolutionary Links Between Metabolism and Immunity

The idea that cytokines – which are the main mediators of immunity and inflammation – can act as hormones, which are the main mediators of metabolism, has been a topic of research for the past three decades. The first cytokines discussed in this context belonged to the interferon (IFN) family. Since then, studies have frequently noted that other cytokines such as interleukin 1 (IL-1), IL-6, and TNF can possess hormonelike properties, thereby influencing the metabolic system. On the other hand, hormones are able to influence the actions of the immune system.

However, "immunometabolism" is a recently emerged era of research that allows the exploration of how the immune system and the metabolic system interact to exert effects beyond the defined physically shared space [11, 13, 14]. The core components of the cross talk between immune and metabolic signaling are highly conserved among species, from flies to mammals [11]. They consist of the following separate signaling pathways.

The Cytokine Tumor Necrosis Factor (TNF) and Its Receptor Signaling

In adipose tissues, the binding of TNF to its receptor on cell membrane is accompanied by the activation of TNF receptor associated factor (TRAF). TRAF acts as an adopter protein responsible for signal transduction from the cell-surface receptors to the nucleus. Then, the activated TRAF stimulates signaling kinases that are involved in the intracellular c-Jun amino-terminal kinase (JNK) and nuclear factor NF-Kappa-B (NF-κB) signaling cascades. Activation of the JNK pathway can diminish the production of insulin (for review, see [15]). Also, the JNK overexpression has been demonstrated to depress phosphorylation of protein kinase B or Akt (PKB/Akt) by insulin. Subsequently, reduced Akt activity results in inactivation of Forkhead box O (FoxO) transcription factors [16] and activation of glycogen synthase kinase-3 (GSK3) [17]. Impaired Akt/FoxO signaling is linked to oxidative stress [18], glucose toxicity [19], adipogenesis, and lipogenesis [11]. GSK3 contributes to a variety of cellular functions favorably glycogen metabolism and inflammation [20, 21]. Animal studies reveal that its targeting offers a potential benefit in immunometabolic disorders [21].

TLR Signaling Pathway

Saturated fatty acids (SFAs) are able to stimulate cell-surface TLR2 and TLR4, which employ the myeloid differentiation primary response 88 (MYD88). MYD88 is an adaptor protein linking TLRs to the activation of JNK and NF-κB signaling pathways. Intracellular events followed with the activation of these pathways were explained above.

Insulin and Its Receptor Signaling

In mammals, the activation of both TNF and TLR signaling pathways appears to mediate insulin resistance. As mentioned above, they share the signal transduction JNK cascade, which, through phosphorylation of the insulin receptor, (IRS) precludes its interaction with insulin [22].

The Evolutionary Links Between Microbiota and Immunity

It is commonly believed that microbes are bad for the human health, as evidenced by the harmful effects of gut bacteria such as gastrointestinal infections, liver problems, and malignancies

[23]. This view is, however, only partly true. There are communities of bacteria, called the human microbiota, homing in various tissues such as the mouth, lungs, intestines, skin, and vagina [24]. The immune host-bacteria interactions are mutualistic; the host immune system plays a role to maintain the homeostasis of the microbiota and vice versa. Generally, the microbiota plays an important role in peripheral regulatory T-cell (Treg) differentiation and T_H-cell differentiation. A precisely defined composition of the microbiota is therefore necessary to maintain T_H17/Treg balance. Otherwise, the immune system will tend to generate abnormal pro-inflammatory or anti-inflammatory responses, which each predisposes the individual to particular series of immune-associated diseases. Of particular interest to the present book is the gut where diet acts directly on the immune system. Indeed, the gut encompasses a complex group of commensal microorganisms – gut microbiota – that are perceived to produce health-promoting effects including digestion of carbohydrates [24], absorption of nutrients, and the development of both the innate and adaptive immune systems [24–26]. Particularly, accounting for 25% of adult gut microbiome, bifidobacteria help with reducing intestinal pH, inhibiting the growth of pathogenic bacteria, synthesis of vitamins, lowering blood ammonia and cholesterol levels, and promoting immunity against tumors and during antibiotic therapy (reviewed in [23]).

Altered Gut Microbiota Associated with Anti-inflammatory Effects

Study of germ-free mice colonized with polysaccharide A (PSA) from a commensal bacterium, *Bacteroides fragilis*, showed that this bacterial molecule specifically employs DCs and major histocompatibility complex (MHC) II molecules to produce immunomodulatory effects by inducing CD4+ T-cell proliferation, secondary lymphoid tissue development, and T-cell-mediated cytokine production [27], whereas lack of PSA altered the T_H1/T_H2 balance resulting in a T_H2 skewing [27], which has been implicated in systemic autoimmune diseases, allergy, asthma, and human immunodeficiency virus (HIV) [28].

Altered Gut Microbiota Associated with Pro-inflammatory Effects

Colonization of the intestine with segmented filamentous bacteria (SFBs) caused pro-inflammatory effects including an augmentation of T_H1 and T_H17 responses and T_H1 cytokine production (IFN-γ and IL-17A) as well as a reduction in the proportion of Treg cells [29]. This SFB-mediated alteration of immune phenotype could explain the observation that as compared to germ-free mice, germ-free mice colonized with SFBs revealed an increased activation of antigen-specific T cells by DCs and consequently were more likely to develop experimental autoimmune encephalitis (EAE) [29] and autoimmune arthritis [30]. It is thus concluded that the gut microbiota effects are not restricted to the gut but can extend beyond the gut – even to the spleen and central nervous system (CNS) – resulting in extraintestinal manifestations.

Chapter 10 has depicted the reciprocal interaction between the gut microbiome and immunity and discussed the potential implications for health promotion and for the management and treatment of human diseases. Meanwhile, the authors have provided evidence supporting the ability of dietary factors, importantly probiotics, to dynamically influence the gut microbiome and immune responses.

Nutrients and Immunity

To prepare sufficient amounts of essential micronutrients for his own needs, the man will have to supplement his food. Studies investigating the effects of deficiency of a single nutrient have concluded that there are nutrients essential to the proper functioning of the immune system, such as vitamin A, beta-carotene, vitamin B9 (folic acid), vitamin B6, vitamin B12, vitamin C, vitamin E, riboflavin, iron, zinc, and selenium [31]. Since deficiency of a single such nutrient can cause immunodeficiency, it is well expected that malnutrition associated with deficiency of multiple nutrients may result in immune-related issues [31].

Vitamins

Vitamins are divided into water-soluble (B-complex vitamins and vitamin C) and fat-soluble (vitamins A, D, E, and K) vitamins. Roughly speaking, biosynthetic pathways of vitamins play a role in signaling microbial infections including bacteria and yeast [32].

Vitamin A

Vitamin A and its metabolites (retinoic acid) are able to contribute to a variety of immune responses such as antibody production; cytokine production; lymphocyte proliferative responses; T-cell effector functions; tumoricidal activation of macrophages, NK cells, and T cells; and mucosal immunity (for review, see [33, 34]). Vitamin A is believed to be the major anti-infective vitamin. Its deficiency – which is supposed to be a serious immunodeficiency condition – breaks down resistance against infectious diseases [35] that would bring high mortality and morbidity. Moreover, maternal vitamin A deficiency is known to predispose to a higher mother-to-child transmission of HIV-1 [36]. Particularly, retinoic acid offers the potential for the regulation of IgA class switching and for the generation of gut-homing B and T cells (reviewed in [37]). It would provoke the expression of gut-homing molecules by B and T cells and thus dictating the gut as where these cells should home in (gut tropism) [38, 39]. Supporting this, T cells and IgA-secreting B cells are absent in mice lacking vitamin A. Supplementation with vitamin A is a cost-effective technique to promote immune system functioning in response to vaccines (tetanus and diphtheria toxoids and measles) and infections [40]. In Chap. 3, Roy and Awasthi review mechanisms through which vitamin A can exert enhancing and modulatory effects on the immune system.

Vitamin B

The book includes two chapters by Mikkelsen and Apostolopoulos who have reviewed the function of each vitamin B in the immune system.

Vitamin B1 (Thiamine)

The ability of vitamin B1 to produce anti-inflammatory effects, control B-cell metabolism, and promote intestinal IgA production is well-documented [41, 42]. In particular, through interaction with peroxisome proliferator-activated receptor γ (PPAR-γ), vitamin B1 is able to activate macrophages and inflammatory responses, and hence, limitation in *Mycobacterium tuberculosis* growth occurs [43]. Thiamine also functions as a cofactor in tricarboxylic acid (TCA) cycle, which provides energy to naïve B cells. It is, therefore, not surprising that its deficiency causes a decrease of naïve B cells [44] concurrent with an impaired induction of antigen-specific IgA responses. In addition, it is known to play an important role in plant resistance by improving antibacterial immunity [45]. The addition of this essential element to rice and vegetable crops has been used to induce resistance to different types of infections including bacterial, fungal, and viral infections [45].

Vitamin B2 (Riboflavin)

Many bacterial and fungal species, including commensal bacteria, produce vitamin B metabolites that acting as antigens improve antimicrobial immunity [32, 46]. Mucosal-associated invariant T (MAIT) cells are a recently identified class of innate-like lymphocytes that exhibit T-cell receptor on their surface and are found in the liver and blood [47]. They are able to recognize vitamin B (vitamin B2 and B9) metabolites presented by the MHC class I-like molecule MR1 and induce the production of cytokines [48]. In this manner, those microorganisms help the improvement of innate immune responses by riboflavin-induced priming of MAIT cells. Dietary depletion of riboflavin would decrease the mRNA expression of antimicrobial peptides and anti-inflammatory mediators and, also, increase the expression of pro-inflammatory mediators, which together lead toward a pro-inflammatory state [49]. On the contrary, it has been shown that administration of vitamin B2 to mice would increase the numbers of neutrophils and monocytes and macrophage activity and thereby make animals more resistant to *E. coli* infections [50]. Moreover, vitamin B2 acts as a cofactor of NADPH oxidase 2 (Nox2) that mediates phagocytosis of *Listeria monocytogenes* by macrophages [51]. Its deficiency results in impaired immunity to *Listeria monocytogenes*.

Vitamin B3 (Nicotinic Acid or Niacin)

Commensal bacteria manufacturers produce this essential vitamin. Also, butyrate is a by-product of dietary fiber fermentation by gut microflora. Niacin and butyrate bring about immunosuppressive effects through binding to a common colonic receptor known as GPR109A that, in turn, can foster the generation of T_{reg} cells and the production of anti-inflammatory cytokine IL-10 in the colon [52]. In this manner, both niacin deficiency and dietary fiber restriction can cause colonic inflammation. Moreover, niacin is a lipid-lowering vitamin that can reduce the concentrations of total cholesterol, triglyceride, very low-density lipoprotein, low-density lipoprotein (LDL), and lipoprotein(a). It is the best available agent to raise high-density lipoprotein levels as well. It is most interesting to us that niacin can protect against vascular inflammation by limiting reactive oxygen species (ROS) content, LDL oxidation, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression, and monocyte and macrophage adhesion in the aorta wall (for review, see [53]).

Vitamin B5 (Pantothenic Acid)

Inadequate production of pantothenic acid by gut microbiota can instruct intestinal cells to enter into an inflammatory state. This vitamin is also essential to the growth of *Lactobacillus plantarum*, which, in turn, play an important role in the synthesis of acetylcholine [54–56]. Acetylcholine functions as the chief neurotransmitter of parasympathetic nervous system. Altogether, pantothenic acid deficiency can intensify inflammation and sympathetic nervous activity [57]. It has also been shown to disturb immune and physical barrier function in fish [58]. Of note, aggravating effect of pantothenic acid deficiency on inflammation is associated with vitamin D deficiency [57].

Vitamin B6 (Pyridoxine)

Evidence indicates the crucial role that vitamin B6 will play in metabolism of lipids, amino acids, and nucleic acids. Therefore, vitamin B6 is a key regulator of cell growth. More interesting is its importance in lymphocyte trafficking (for review, see [37]). It has been shown to act as a cofactor for sphingosine-1-phosphate (S1P) lyase, which is involved in the degradation of S1P. S1P is a bioactive lipid mediator that its gradient governs cell trafficking. A 20-day period of vitamin B6 depletion resulted in a reduction in the numbers and percentage of lymphocytes as well as evoked immune responses as determined by cytokine (IL-2) production and mitogenic responses of peripheral blood lymphocytes [59], whereas a 14-day course of vitamin B6 supplementation could increase the number/percentage of T-lymphocyte cells, T-helper cells, and T-suppressor cells in critically ill patients [60]. Moreover, the ratio of T4 lymphocytes (helper cells)/T8 lymphocytes (cytotoxic and suppressor cells) was markedly elevated by supplementation of vitamin B6. This effect is of special importance to patients with HIV who reveal a reduction in the ratio of T4/T8 lymphocytes [61].

Vitamin B9 (Folate or Folic Acid)

Vitamin B9 contributes to the synthesis of proteins and nucleic acids. Its deficiency has been implicated as a causal factor in anemia, liver diseases, cardiovascular diseases, birth defects, and cancer. In general, the need for folate is more pronounced in cell-mediated immunity than in humoral immunity. Particularly, folate has been demonstrated to support the expression of anti-apoptotic Bcl-2 protein and thereby assist T cells to survive as Treg cells [62, 63]. Treg cells, in turn, express folate receptor 4. Consequently, B9 depletion would decrease the number of Treg cells in both the small [62] and large [63] intestine, conferring an increased susceptibility to intestinal inflammation. Also, folate appears important for proliferative response of cytotoxic CD8 + T cells [64]. Along with megaloblastic anemia, folate deficiency would result in the S-phase arrest, upregulate uracil level in DNA, and ultimately induce apoptosis. It is immunologically characterized by a reduced ratio of CD4+/CD8+ T cells due to a reduced proliferation of cytotoxic CD8 + T cells.

Vitamin B12 (Cobalamin)

Like vitamin B9, vitamin B12 is preferentially attached to cell-mediated immunity rather than humoral immunity. Patients with vitamin B12

deficiency anemia are characterized by immunological abnormalities including lower numbers of lymphocytes and CD8+ cells, lower proportion of CD4+ cells, higher ratio of CD4+ to CD8+ T cells, and diminished NK cell activity [65]. Interestingly, B12 treatment has been shown to successfully restore all these parameters.

Vitamin C

Vitamin C serves as both immunostimulant and immunomodulator. Supplementation of vitamin C would enhance the innate immune parameters, e.g., lysosomal function, respiratory burst activity, and complement activation [66]. It can also improve cell-mediated immunity by reinforcing T-cell proliferative response [67] while diminishing the production of pro-inflammatory cytokines (IL-6 and TNF-α) in lipopolysaccharide-induced monocytes [68]. It can be used as a means to enhance immunity against infections [69, 70]. Chapter 5 provides detailed discussion of the relationship between vitamin C and immune homeostasis.

Vitamin D

Apart from skeletal effects, vitamin D possesses pleiotropic effects including immunomodulatory and antiproliferative actions [71]. Therefore, vitamin D deficiency is considered a contributing factor to varieties of health issues, including musculoskeletal disorders, infections, autoimmune diseases, cardiometabolic diseases, cancers, cognitive impairment, mental disorders, and infertility [72]. As its receptor is expressed in immune cells such as lymphocytes, DCs, and monocytes, vitamin D has sufficient capacity to affect different immune processes [72]. Modulatory effects of vitamin D on the adaptive immune system are of special benefit to patients with autoimmune diseases, e.g., multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [73]. Also, supplementation with vitamin D would improve innate antimicrobial immunity. More precisely, it has been shown to upregulate the IFN-γ signaling pathway and therefore the phagocytosis ability of macrophages, which together help the immune system to more effectively control *Mycobacterium tuberculosis* [74]. Vitamin D insufficiency/deficiency is a major global health problem [75] with greater than 40% prevalence in Europe [76]. Besides dietary supplements, sunlight is of great importance to supply vitamin D. The next chapter will discuss about the role of vitamin D in the immune system in more detail.

Vitamin E

An optimal level of plasma vitamin E is necessary to generate mature T cells in thymus [77] and to maintain B- and T-cell responses to mitogens [22], particularly in the elderly [77]. Supporting this, supplementation with vitamin E would reduce prostaglandin E2 (PGE2) synthesis and lipid-peroxidation products and thereby boost cell-mediated immune responses as reflected in an increased delayed-type hypersensitivity (DTH) in aged people [78, 79] and animals [80]. Additionally, supplementation with vitamin E can bring about an increase in humoral immune responses such as antibody production, macrophage activity, and phagocytosis [81, 82]. A study of elderly people revealed that the higher the plasma levels of alpha-tocopherol (a type of vitamin E), the lower the risk of infections in the previous 3 years [83]. More interesting is that vitamin E intake above the recommended allowance continues to be safe for elderly patients while enhancing immunity [84]. Overall, it provides a strategy to resist against infections [85] and to delay the progression to AIDS [86].

Minerals

Selenium

Generally, selenium makes a vital contribution to both the innate and adaptive immunities. In particular, it is important to lymphocyte proliferative response, neutrophil function, and antibody production [87]. This essential element in the form of amino acid selenocysteine actively takes part in the structure of selenoproteins [87]. Glutathione peroxidases are a class of selenoproteins particularly involved in the cross talk from oxidative stress to immune responses [88].

Chapter 9 cover the role of selenium in the context of immunity.

Zinc

Zinc is a transition metal that its capacity to directly participate in the structure of many proteins makes it essential for the human body. Invading pathogens, such as bacteria and viruses, use transition metals to promote their metabolism and therefore thriving within the host [89]. Immune function compromised by zinc deficiency is serious such that it is considered as an immunodeficiency condition. Immune cells particularly sensitive to zinc deprivation are T cells, naïve B cells, NK cells, monocytes and macrophages, mast cells, neutrophils, and DCs (for review, see [90]). Consequently, zinc deprivation can cause a shift to T_H2 cytokine response, a reduction in antibody generation, decreased killing activity, reduced phagocytosis, decreased Fc-receptors-dependent mast cell activation, reduction in neutrophil number and recruitment, and decreased LPS-induced DC activation. As reviewed in [91], zinc supplementation is beneficial in treatment and prevention of various diseases including infections. It is worth mentioning that the effect of zinc supplementation can occur at any stage of life and in medical settings as well. For instance, study shows that elderly people and also patients with sickle cell disease benefit from lower infection rates linked to zinc supplementation. In children, zinc supplementation was effective in reducing respiratory tract infections. Chapter 8 by Gammoh and Rink describes the role of zinc and its signaling on innate and adaptive immune functions. The chapter also includes a part on the effects induced by zinc deficiency in certain susceptible populations.

Immunometabolic Disorders

All people need nutrients to supply energy and thrive under selective pressures. The level of intake should be managed as the pressure changes. Otherwise, when the pressure is relatively small, metabolic disorders are most likely to develop if food intake is held constant. Immune pathways are the key reason that nutrients essential for living can be damaging, making them a modifiable risk factor for human health and therefore interesting targets for the emerging field of immunometabolic disorders [11]. Besides obesity, other physiological/pathological situations associated with nutritional abnormalities as well as immune impairment include protein-energy malnutrition (PEM), aging, obesity, eating disorders, sports requiring low body weight, food allergy, and gastrointestinal disorders (for review, see [31]). Below is a rapid overview of immunometabolic disease clusters covered in the book.

Obesity and Diabetes

More than 30% of the adult population in the United States are estimated to be obese [92, 93]. Obesity is, thus, considered as the most common nutritional disorder of adulthood. Inflammation ranks at the top of factors contributing to the link between obesity and type 2 diabetes (T2D). More precisely, the progression from lean to obese is a high-risk process fraught with pro-inflammatory effects, which occur particularly in macrophages, mast cells, $CD4^+$ T-helper 1 (T_H1) cells, and $CD8^+$ effector T cells. In contrast, it inhibits anti-inflammatory functions of eosinophils, T_H2 cells, and T_{REG} cells. Overall, the obesity-associated inflammatory events will be synchronized to produce a chain of metabolic changes that precede the onset of other immunometabolic diseases [94].

Chapter 18 shows the effects of the cross talk between adipose tissue and immune cells on obesity.

Cancer

Recent research stresses the importance of environmental factors rather than inherited genetic mutations in carcinogenesis [6]. Environmental factors, especially dietary factors, through epigenetic modification can cause inflammation, allowing a tissue microenvironment to develop cancer [6]. Even low-grade chronic inflammation as part of obesity makes the body more susceptible to cancer [6]. Inflammation is not only involved in initiation but also in tumor progression and metastasis [6]. Nutrients with antioxidative and immunomodulatory properties that

interfere with carcinogenesis include vitamins A and C, zinc, selenium, probiotics, and polyunsaturated fatty acids (PUFAs) [95].

The book includes two chapters tracing the links between nutrition, immunity, and cancer.

Neurodegeneration and Aging

Chronic subclinical inflammation undermines optimal functioning of the brain [96] through upregulation of cytokine networks contributing to neurodegeneration and aging [97]. Deficiency of essential nutrients, especially vitamin D, vitamin B6, vitamin C, vitamin B1, vitamin B2, and iron, is common among aged, even in multivitamin-supplemented people [98] and might account for the increased risk of infections and related mortality and morbidity in the aged population. Results from epidemiological studies are promising that there is a broad range of micronutrients and macronutrients important for protecting the brain from cognitive dysfunction [99]. A proposed mechanism is based on the inhibition of vascular inflammation that has implications for aging and different associated diseases [100].

In Chap. 14, Di Benedetto and Müller examine how nutrient factors and immune responses interact to modulate neuroinflammation and aging. Meanwhile, Patel investigates the potential of nutritional programming for improving the body's homeostasis including the neuroimmune system.

Asthma, Allergies, and Inflammatory Diseases

The anti-inflammatory effects of nutrients are well-documented. It is, therefore, not surprising that cells will undergo inflammation when nutrient levels are low, and it can be enough for the susceptible tissues to develop asthma, allergies, and inflammatory diseases. In fact, dietary factors directly influence the composition of microbes residing in the intestinal and extraintestinal tissues, which, in turn, actively take part in the generation of immune responses [101].

In Chap. 15, the authors will particularly discuss the role of vitamins C, D, and E and their implications in the context of immunity to asthma. Also, Chap. 16 gives a general overview of food intolerances. It is followed with Chap. 17 covering detailed information on T cells and their potency in food allergy treatment. Chapter 21 studies the effects of nutrition and foods on autoimmune diseases as well.

Immune Programming

Genetics, Nutrition, and Immunity

The emerging field of nutrigenomics has helped understanding of the influence that genetic polymorphisms of immune mediators leave on metabolic pathways on one side and, on the other side, made tangible how nutrition and dietary factors can alter the expression of genes involved in immune responses [102]. Chapter 24 provides a detailed synthesis of studies searching for nutrigenomic immunity.

Epigenetics, Nutrition, and Immunity

Unlike genetic factors, epigenetic marks are reversible, allowing reprogramming after birth. The ability of epigenetic modifications to mediate the effect of nutrition on the body's homeostasis throughout life from embryogenesis to aging has attracted a huge interest especially during the last decade. Consequently, nutrition-immunity cross talk through epigenetic regulation is now offering opportunities for prevention and treatment of human diseases from chronic noncommunicable diseases to brain and behavioral disorders. Chapter 25 addresses how the offspring epigenome would be influenced by maternal diet, with a brief discussion on nutriepigenomic immunity.

Conclusions

In this chapter, we introduced the immune system and its innate and adaptive arms. Energy balance is the key to immune homeostasis. Also, there are evolutionary links between nutrition and immunity that mainly involve signaling by

cytokines (TNF), innate immune receptors (TLRs), and insulin. Of particular importance is the gut where nutrition and foods directly affect the composition of the gut microbiota, which, in turn, would influence the immune homeostasis. Therefore, dietary factors through modification of the gut microbiota may cause pro-inflammatory effects throughout the body, thereby resulting in inflammatory disorders, collectively called immunometabolic disorders. In this manner, the Book is finished by pointing out that the effect of nutrition on immunity is not superficial but runs deep to the genome and epigenome, and therefore, it is not temporary but can be transferred to other lineages.

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Vitamin D and the Immune System

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Key Points

- Vitamin D plays role in homeostasis of calcium and phosphate and therefore is necessary to maintain metabolic and skeletal health.
- Vitamin D interacts with immune cells expressing its receptor and thereby influencing innate and adaptive immune responses.
- Vitamin D serves as a potent stimulant of innate defense, while it is thought to be a tolerogenic immunomodulator in adaptive immunity.
- Recent studies support vitamin D as a promising and safe nutrient for prevention and adjunctive treatment of several immune-associated disorders.

Introduction

Vitamin D (VitD) is a secosteroid hormone, originally recognized for its pivotal role in mineral metabolism and skeletal health through homeostasis of calcium and phosphate. In the early 20th century, the important finding that rickets in children and osteomalacia in adults are associated with lack of VitD brought this compound to the attention of the scientific community. This led to the understanding of skeletal functions of VitD and also a significant drop in prevalence of rickets and osteomalacia in many parts of the world. It is now well known that VitD increases intestinal calcium and phosphate absorption, induces the renal reabsorption of calcium in the distal tubules, stimulates osteoclast activation and thereby calcium reabsorption from the bones, and enhances mineralization of the collagen bone matrix through maintaining adequate calcium and phosphate levels. However, the effects of VitD on the human body are much wider than its classical role in skeletal homeostasis.

A growing body of literature over the past few decades has demonstrated the diverse effects of VitD in several extra-skeletal systems and their related disease states. The paradigm-shifting finding that almost every tissue in the body expresses the VitD nuclear receptor and thus responds to the function-modulating effects of VitD at a cellular level reshaped the perspective on how vitamin D influences human health. Microarray analyses show that up to 5% of the human genome is directly or indirectly regulated by VitD [1]. Studies indicate that VitD interferes with more than 160 biological pathways in 36 different cell types [1]. Moreover, many epidemiologic studies have linked low VitD status to a variety of pathologic conditions in many different organs and systems in the human body, including cancer, cardiovascular diseases, metabolic syndrome, neurological conditions, and – of greatest relevance for this chapter – several major immune-related disorders and infectious diseases.

Technically, the association between VitD and immunity and infection has long been appreciated, without the underlying mechanisms being clearly understood. Before development of effective antibiotics, VitD was used – unknowingly – to treat infectious diseases, particularly tuberculosis (TB). It is now known that VitD can be endogenously produced after skin exposure to ultraviolet (UV) solar irradiation. VitD was initially referred to as the "sunshine cure" since it mimicked the effects of sun exposure on TB patients. According to the writings of Hippocrates, solar therapy has been in use to treat infectious disorders, particularly TB, at least since the ancient Greek era. In the eighteenth- and nineteenth-century Europe, heliotherapy (sun exposure) in sanatoriums or open-air sun-exposed mountain retreats was a common practice for treatment of TB. The 1903 Nobel Prize in medicine was awarded to Niels Finsen for his demonstration that UV light was beneficial in treating cutaneous TB. Moreover, cod liver oil – a rich dietary source of VitD with more than 1000 International Units (IU) of VitD per tablespoon [2] – has traditionally been employed for treatment of TB patients, chronic rheumatism, and general protection from infections. The first scientific evidence for the efficacy of cod liver oil supplementation was provided as early as 1848 at the Brompton Hospital in London, when a clinical trial of cod liver oil in 542 TB patients reported a significant inhibition of disease progression. Cod liver oil supplementation became a popular anti-TB practice for almost a century,

during which steady drops in TB-related death rates were reported in the UK [3]. In the modern era, evidence for the direct involvement of VitD in the immune system emerged in the 1980s, when, for example, a 1986 study showed that VitD inhibits the growth of *Mycobacterium tuberculosis* in cultured human macrophages [4]. Since then, interest in elucidating the role of VitD in the immune system has resulted in the accumulation of a vast body of evidence that, as will be discussed in this chapter, indicates the deep involvement of VitD in human innate and adaptive immune responses.

Metabolism of VitD

Sources and Synthesis

A vitamin is defined as a substance present in minute amounts in food and is essential to consume to avoid pathology. Although widely viewed as a vitamin, VitD is in fact a secosteroid hormone, derived from both endogenous and nutritional sources (Fig. 2.1). Cholecalciferol (D_3) and ergocalciferol (D_2) represent the two major biological precursors of VitD. In humans, the main source of D_3 is the endogenous cutaneous synthesis of D_3 from 7-dehydrocholesterol through a photochemical reaction upon exposure to the solar UV-B irradiation (wavelength 290–320 nm). Exogenous sources of D_3 are limited to fatty fish (e.g., salmon, mackerel, sardines, tuna), fish liver oil, egg yolk, as well as VitD-fortified foods and supplements. D_2 is mostly found in VitD-fortified foods and supplements, but certain sun-dried mushrooms also contain D_2. Both D_2 and D_3 circulate the blood as inactive prohormones bound to VitD-binding protein (DBP) and require two sequential hydroxylations to become biologically active, after which they appear to have comparable biological activity. The first hydroxylation is accomplished in the liver, where through the action of cytochrome P450 25-α hydroxylase enzymes such as CYP2R1 and CYP27A1, 25-hydroxyvitamin D (25(OH)D, calcidiol or calcifediol) is formed. With a half-life of 2–3 weeks, 25(OH)D is the main circulating form of VitD in the body, and as will be discussed later, measurement of its serum levels is the most commonly used method to define human VitD status. Most 25(OH)D circulates in the blood bound to DBP as an inactive metabolite (80–85%), with a smaller portion less strongly bound to albumin and a minute portion freely circulating in the blood. 25(OH)D is then hydroxylated again, mainly in the kidney proximal tubule cells, by the cytochrome P450 1-α hydroxylase enzyme CYP27B1, thus forming $1,25(OH)_2D$ or calcitriol, which is the hormonally active form of VitD in the human body. The second hydroxylation is stimulated by the parathyroid hormone (PTH) and inhibited by the calciuric hormone fibroblast growth factor-23 (FGF-23). Moreover, calcitriol levels are strictly regulated by a renal negative feedback mechanism, where high serum calcitriol levels inhibit CYP27B1 and stimulate CYP24A1 (24-α hydroxylase) which initiates catabolic degradation of calcitriol into the inactive, water-soluble form, calcitroic acid, which is then excreted in the bile. $1,25(OH)_2D$ is also transported in the blood bound to DBP; however, it has a relatively short plasma half-life of only hours.

Although the kidneys are the main site of $1,25(OH)_2D$ production, it is crucial to note that they are not the only source. CYP27B1 is present and active in many extrarenal tissues including breast, prostate, bone, brain, smooth muscle, as well as the immune system. Many immune and inflammatory cell types can convert the circulating 25(OH)D into calcitriol including monocytes, macrophages, dendritic cells, and activated lymphocytes. Unlike renal generation of calcitriol, the activity of 1-α hydroxylase CYP27B1 in the immune cells is not regulated by serum PTH, calcium, and calcitriol levels; in contrast, it is mostly stimulated after exposure to local inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1, and lipopolysaccharides (LPS). In other words, extrarenal CYP27B1 is nonresponsive to systemic regulators and is induced by local factors. In conclusion, it seems that in addition to the $1,25(OH)_2D$ endocrine renal loop, which provides constant levels of *circulating* calcitriol that mostly affect calcium and phosphate homeostasis, VitD signaling also involves paracrine and autocrine pathways that provide high *local* concentrations of calcitriol in

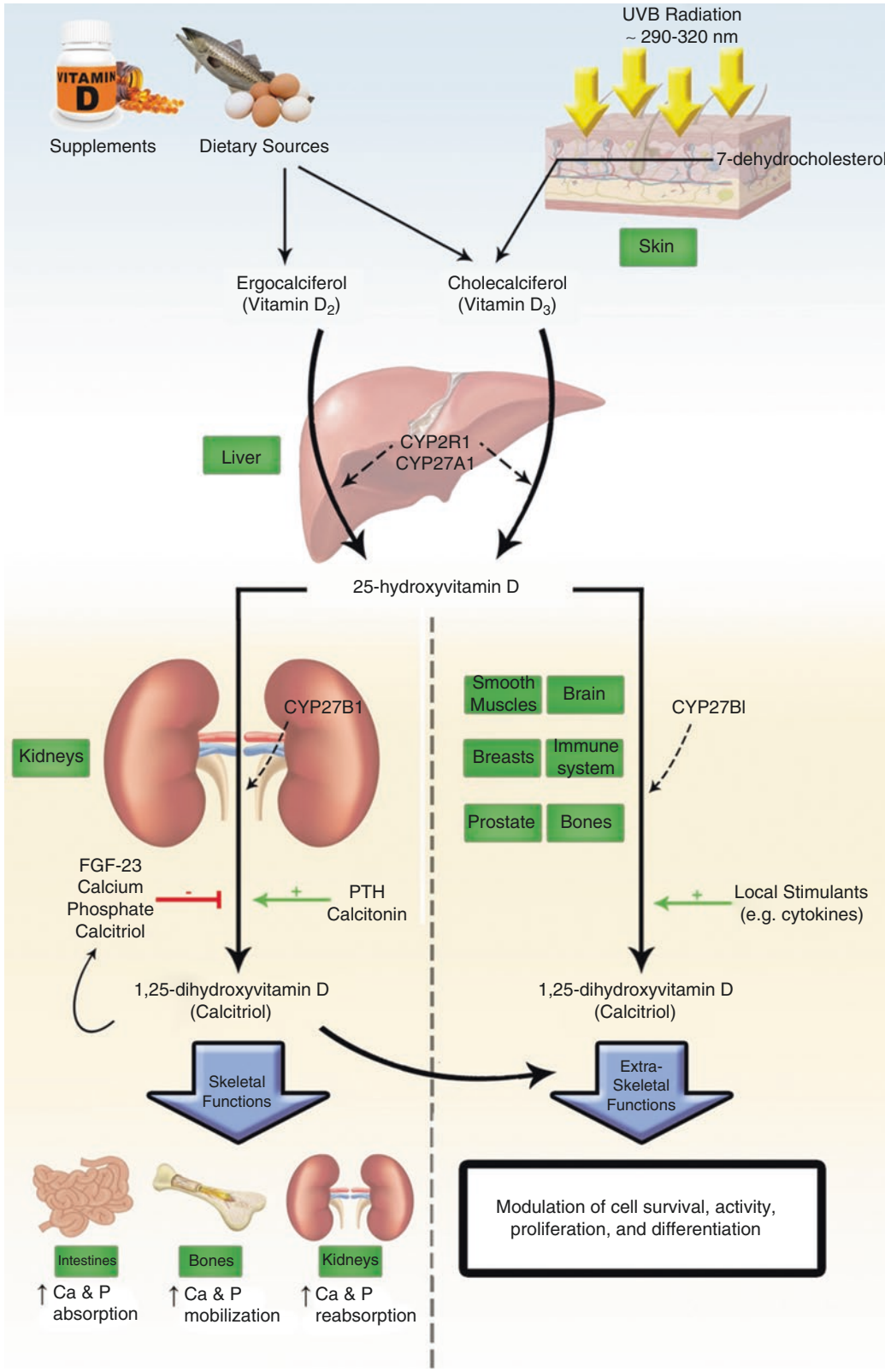

Fig. 2.1 Synthesis and metabolism of VitD. Ca calcium, P phosphate, PTH parathyroid hormone, FGF-23 fibroblast growth factor 23

various tissues throughout the body, affecting cell growth, differentiation, proliferation, and several other cellular functions in many cell types.

VitD Receptor

Like other steroid hormones, the biological effects of calcitriol are mediated through a nuclear secosteroid receptor. VitD receptor (VDR) is activated upon the binding of calcitriol to the α-helical ligand binding domain of the VDR. The calcitriol-VDR complex acts as a transcription factor after dimerizing with the DNA-binding protein retinoid X receptor (RXR). The calcitriol-VDR-RXR heterodimer binds to several specific regulatory sequences within the promoter region of the target genes, termed VitD-responsive elements (VDREs). This eventually results in the modulation of transcription and expression of specific gene products [5, 6]. Besides acting as an independent transcription factor, the calcitriol-VDR complex can also modulate the expression of target proteins through binding to other transcription factors (e.g., STAT-1 and IKK-β) [7]. Interestingly, recent studies have brought to light the presence of non-nuclear receptors for VitD. These distinct VDRs are located at the cell surface and perinuclear area and are collectively termed as membrane VDRs (mVDRs). VitD can exert non-genomic rapid biological effects through binding mVDRs, which subsequently activates several intracellular signaling pathways [8]. As mentioned earlier, VDR is expressed and active in a multitude of cell types present in the human body and regulates hundreds of biological pathways through the described mechanisms. In the immune system, VDR is constitutively expressed in monocytes, macrophages, and dendritic cells and inducibly expressed by the lymphocytes upon activation [9].

Serum 25(OH)D Levels

Defining VitD Status

As was mentioned earlier, considering the very short half-life, extremely low serum concentrations, and highly lipophilic nature of calcitriol, serum levels of 25(OH)D are the principal marker used for assessment of VitD status. There is little consensus on the 25(OH)D serum level threshold that most adequately distinguishes biological VitD deficiency, insufficiency, or sufficiency, meaning that there is no common definition for adequate VitD status. The Institute of Medicine defines VitD *deficiency* in adults as serum 25(OH)D concentrations of less than 30 nmol/l (12 ng/ml) and considers serum levels of 50 nmol/l (20 ng/ml) and higher as *adequate* [10]. In contrast, the Endocrine Society and International Osteoporosis Foundation define *deficiency* as levels below 50 nmol/l (20 ng/ml), and *sufficiency* as levels of 75 nmol/l (30 ng/ml) and higher [11, 12]. Consensus will be difficult given the likelihood that different populations may have different levels due to different levels of DBP and that different levels of 25(OH)D may mean different things depending on the outcome (disease) of interest.

Factors Affecting VitD Status

Low VitD status could be regarded as a pandemic as it was estimated to affect more than one billion people worldwide [13]. Many population-wide and individual factors affect VitD status. In a person not under treatment with VitD supplements, only a minor portion of the VitD needs are derived from dietary sources, except in case of rare dietary habits. Therefore, VitD status of a subject mainly depends on endogenous UV-B-mediated synthesis of D_3. As a result, the levels of VitD are affected by factors like clothing, latitude, altitude, season, cloud cover, air pollution, skin pigmentation, skin health, and lifestyle (e.g., indoor vs. outdoor, use of sunblock), all of which collectively determine the amount of UV-B irradiation the epidermis receives. For example, at 45° latitude, which is south of many major population centers of Europe and North America, the radiation intensity is too low to ensure sufficient VitD synthesis for almost 6 months of the year [14]. This so-called VitD winter is even longer in areas with higher latitudes. Higher rates of VitD deficiency in dark-skinned African or African-American populations is another example, which

arises from the higher absorption of UV-B by the cutaneous melanin and therefore lower availability of UV-B for VitD synthesis. Compared to light-skinned (white) populations, dark-skinned subjects need six times more UV-B exposure to reach the same serum 25(OH)D levels [15]. Serum 25(OH)D levels in African-Americans have been shown to be approximately one-half those of white (European) Americans [16]. It is also known that serum 25(OH)D levels are at their lowest levels after winter and reach their maximum at the end of summer, thus reflecting the seasonal variation in VitD status [17]. As was discussed earlier, there are very few natural non-fortified foods that contain relevant amounts of VitD. Therefore, the regional food fortification strategy is another significant contributing factor to VitD status in a population. Some countries routinely fortify some staple products such as dairy products. Consequently, place of residence and nutritional habits are important parameters that affect individual vitamin D dietary intake. Furthermore, sufficient amounts of 7-dehydrocholesterol are needed for D_3 synthesis. Elderly people are often found to be VitD deficient due to structural changes of skin and limited bioavailability of 7-dehydrocholesterol. Cutaneous synthesis of VitD in individuals over 70 years old is half that of younger than 20 subjects in otherwise similar conditions [18].

VitD Supplementation

Considering that several individual factors, described above, influence VitD status of a subject, and in the absence of commonly approved guidelines for target serum 25(OH)D levels, there is no international consensus on optimal level of VitD supplementation. While many groups have recommended higher daily allowances, the 2010 Institute of Medicine report recommended 400 IU of supplemental VitD per day for birth to 12 months, 600 IU daily for ages 1 through 70, and 800 IU daily for people older than 70 [19]. Supplementation could be in the form of either D_2 or D_3, as administration of the biologically active metabolite calcitriol or its analogs might be associated with potential side effects especially hypercalcemia, and is reserved for particular indications such as chronic kidney disease and hypoparathyroidism. Limited evidence suggests that D_3 supplementation might offer superior efficacy in improvement of serum 25(OH)D levels compared to D_2; however, whether this translates into improved VDR engagement and target cell bioavailability is subject to ongoing debate [20].

As mentioned earlier, many countries use food fortification strategies to prevent VitD deficiency at the community level. VitD fortification of fluid milk is mandatory in the United States and Canada, through which 350–450 IU of VitD is provided per liter of fortified milk. Other commonly fortified products include yogurt, cereal, juice, and cheese, all of which usually contain 40–100 IU VitD per regular serving. As human milk is a poor resource of VitD (given the low vitamin D status of most mothers), Food and Drug Administration (FDA) requires infant formula to contain 40–100 IU VitD per 100 kcal. VitD intoxication – observed only when serum 25(OH)D levels are beyond 375 nmol/l (150 ng/ml) – is a very rare condition characterized by hypercalcemia, hypercalciuria, urinary calculi formation, and calcifications in different organs [21]. To avoid VitD intoxication from supplement use, the Endocrine Society and the Institute of Medicine, respectively, recommend tolerable upper daily limits of 10,000 and 4000 IU for VitD supplementation [10, 12]. However, the therapeutic window seems to be much wider, as most cases of VitD intoxication are associated with prolonged involuntary intake of doses of supplemental VitD as high as 40,000 IU per day or more [22]. These very unusual cases are typically due to ingestion of food that was mistakenly fortified with excess vitamin D, which can be prevented through more rigorous oversight of the food industry.

VitD and the Immune System

Over the past few decades, countless studies have explored the diverse role of VitD in different aspects of the human immune response. Two key concepts contributed most to the new era of VitD as a potential immunomodulator: (a)

Expression of VDR by several immune cell types. As mentioned earlier, VDR is constitutively expressed in neutrophils, monocytes, macrophages, and dendritic cells (DCs) and inducibly expressed by T and B lymphocytes upon activation. VDR modulates the function of up to 500 VitD-responsive genes involved in activation, differentiation, and proliferation of immune cells. (b) *Production of active VitD by several immune cell types.* Unlike the previous assumption, it is now known that the synthesis of $1,25(OH)_2D$ is not restricted to the kidneys. Monocytes, macrophages, and DCs express both 25-α and 1-α hydroxylase enzymes, which enables them to convert serum D_3 or D_2 into 25(OH)D and then $1,25(OH)_2D$. Lymphocytes only express 1-α hydroxylase and are therefore able to convert 25(OH)D into $1,25(OH)_2D$ [23]. It is important to underline that the activity of CYP27B1 in the immune cells is not influenced by the classical endocrine feedback mechanisms. In contrast, the generation of and response to VitD in the immune system involves intracrine and paracrine pathways that are subject to local modulatory signals. Inflammatory stimuli such as IFN-γ, TNF-α, IL-1, IL-2, and LPS upregulate the expression of CYP27B1 through activating the transcription factor C/EBPβ and consequently induce the synthesis of active VitD (calcitriol) provided that the substrate 25(OH)D is sufficiently available [24]. In the chronic granulomatous disease sarcoidosis, excessive serum levels of calcitriol and calcium are detected, owing to the unchecked chronic CYP27B1 activity in alveolar macrophages that are not responsive to serum calcitriol, PTH, and calcium levels [25]. In summary, these two (bolded) concepts clearly establish that immune cells are "wired" – i.e., have the necessary machinery – to directly synthesize and respond to VitD and thus support an immunomodulatory function for VitD similar to well-known inflammatory cytokines.

The evidence for direct involvement of VitD in the immune system was first provided in the 1980s when, for example, a 1986 study showed that active VitD inhibits the proliferation of *Mycobacterium tuberculosis* in cultured human macrophages [4]. Since then, the newfound interest in elucidating the role of VitD in the immune system has resulted in the accumulation of a vast body of evidence that, as will be discussed shortly, indicates the deep involvement of VitD in human innate and adaptive immune responses. The available evidence can be categorized into two groups. The first group, discussed in the current section, comprises of in vitro, animal, and clinical mechanistic studies which explore the specific roles of VitD in a variety of immune cells. Discussed in the following sections are the observational and interventional human studies that try to link impaired VitD status with dysregulation of immune responses and higher prevalence, incidence, and severity of immune-related and infectious disease conditions.

VitD in Innate Immunity

Innate immunity provides the first line of defense against external challenges and prevents spread and exacerbation of infection through rapid recognition and elimination of invading pathogens. The innate immune system consists of a combination of physical and chemical barriers. Monocytes and macrophages are key effector cells of innate immunity and express both VDR and CYP27B1, as mentioned earlier. In addition to phagocytosis of pathogens, their function also involves activation of pattern-recognition receptors including toll-like receptors (TLRs) located on their cell membrane. Upon exposure to pathogen-associated molecular patterns, TLRs initiate a cascade of cellular events aimed for pathogen killing and induction of inflammation, among which is the production of antimicrobial peptides (AMPs) such as α-defensins, β-defensins, and cathelicidin. AMPs are among the first responders of the innate immune attack against pathogens. Human cathelicidin (hCAP18) is the main AMP in the innate immune system with broad microbicidal activity against bacteria, viruses, and fungi. It is encoded by the cathelicidin antimicrobial peptide (CAMP) gene which is expressed in neutrophils and monocytes, as well as DCs, lymphocytes, natural killer (NK) cells, and epithelial cells of the skin, gastrointestinal tract, and respiratory tract [26]. AMPs co-localize with the ingested pathogens within phagosomes and contribute to microbial killing

via destabilization of microbial membranes. Aside from their direct microbicidal role, AMPs modulate many other immune processes, including mast cell degranulation, cell differentiation, vascular permeability, wound healing, and the process of antigen presentation. They also might act as chemoattractants for neutrophils and monocytes and modulate the production of cytokines and chemokines [27–32].

It is well-established that VitD is a stimulator of innate immune response through several mechanisms (Fig. 2.2). Several studies have elucidated the crucial role of the autocrine $1,25(OH)_2D$ pathway in promotion of antimicrobial response through inducing production of AMPs in a variety of cell types. Direct regulation of AMPs by VitD is evidenced by the fact that promoters of human cathelicidin and β-defensin 2 genes, respectively, contain three and one VDREs [33, 34]. Human expression profiling studies have revealed that in monocytes and macrophages, activation of TLR2/1 upon recognition of pathogen antigens strongly induces the expression of VDR and CYP27B1. When sufficient concentrations of circulating 25(OH)D are available, this leads into significant localized production and activation of $1,25(OH)_2D$. Subsequently, the calcitriol-VDR-RXR complexes bind to the VDREs within the promoter of AMP genes, which upregulates the transcription of these genes and expression of AMP proteins, such as cathelicidin, and thereby promotes intracellular microbial killing in phagocytic vacuoles [16, 34, 35].

Various in vitro and in vivo studies have corroborated the explained involvement of calcitriol in induction of AMP production in monocytes and macrophages. Exogenous $1,25(OH)_2D$ has been shown to inhibit the proliferation of *Mycobacterium tuberculosis* in cultured human macrophages [4]. Adams et al. showed that TLR2/1 stimulation of human monocytes by the TLR2/1 ligand 19 kDa lipopeptide resulted in a 5.0-fold increase in expression of CYP27B1 by monocytes. Moreover, expression of cathelicidin correlated with 25(OH)D levels in serum culture supplements and was significantly enhanced by exogenous 25(OH)D [36].

VitD is the key effector that links TLR activation to cellular antimicrobial response, as it is the primary inducer of AMP genes. As explained, since transcription of cathelicidin is absolutely dependent on sufficient levels of VitD, variations in VitD status of individuals seem to affect the induction of cathelicidin expression in cases of infection. A cross-sectional study showed that VitD deficiency in septic patients was associated with lower concentrations of cathelicidin [37]. In another study, VDR-driven induction of CAMP expression in serum-cultured human macrophages was strongly dependent on serum 25(OH)D concentrations. Macrophages cultured in sera from VitD-insufficient individuals were inefficient in inducing the expression of cathelicidin mRNA [16]. Conversely, supplementation of VitD-insufficient individuals has been found to restore monocyte cathelicidin induction following TLR activation ex vivo [36]. These findings provide a rationale for the possible association of VitD deficiency with increased susceptibility to infections.

Neutrophils are a key component of the innate immune response especially in severe infections, and neutrophil granules are known to be a major source of cathelicidin. As indicated earlier, although neutrophils express VDR, activity of CYP27B1 in neutrophils has not been reported. Therefore, unlike monocytes and macrophages, VitD-induced regulation of CAMP in neutrophils – if existent – relies on circulating $1,25(OH)_2D$ produced by kidneys, rather than local intracrine loops [38].

Besides regulation of CAMP, calcitriol is responsible for regulation of other AMPs such as human β-defensin 2 (DEFB4). While $1,25(OH)_2D$ alone is sufficient for strong induction of CAMP expression, it seems that this is not the case in regard with DEFB4. Transcription of DEFB4 also depends on binding of NF-κB to specific response elements within DEFB4 proximal promoter. $1,25(OH)_2D$ propagates the TLR-induced activation of the IL-1β signaling pathway, which results in production and translocation of NF-κB to its DEFB4 binding sites, thereby inducing the expression of DEFB4 in monocytes [33]. Consistent with these findings, studies have

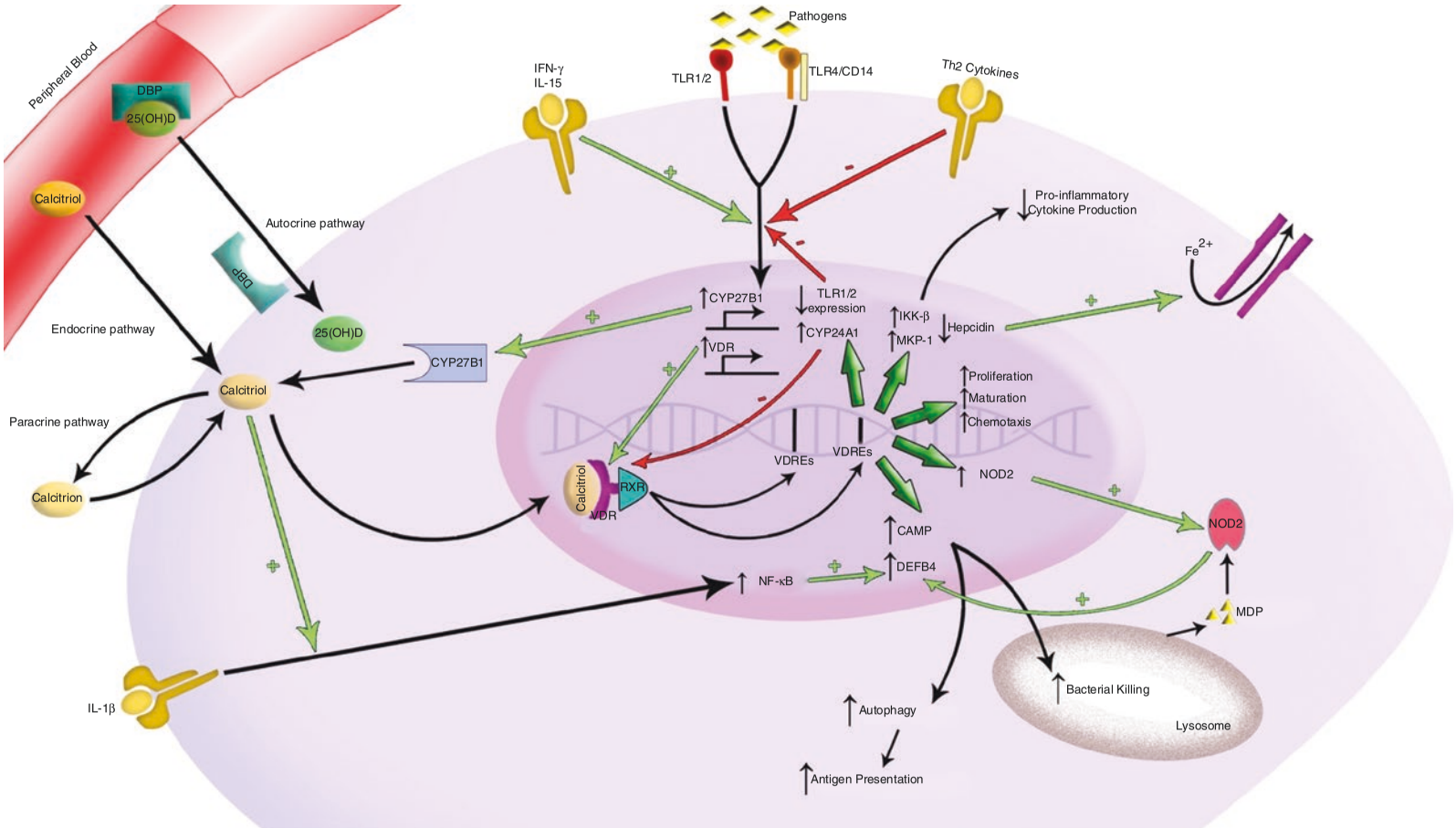

Fig. 2.2 VitD in innate immunity. DBP VitD-binding protein, IL interleukin, IFN interferon, TLR toll-like receptor, Th T-helper, NOD nucleotide-binding oligomerization domain-containing protein 2, MDP muramyl dipeptide, VDR VitD receptor, IKK I kappa B kinase, MKP mitogen-activated protein kinase phosphatase, VDRE VitD-responsive element, CAMP cathelicidin antimicrobial peptide, NF-κB nuclear factor-kappa B, RXR retinoid X receptor

found that isolated calcitriol had modest or even nonexistent effects on the expression of DEFB4; however, $1,25(OH)_2D$ enhanced the strong induction of DEFB4 by IL-1ß by twofold, indicating that calcitriol and IL-1ß are both required for strong induction of DEFB4 [16, 39]. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) represents another class of pattern-recognition receptors. Activation of NOD2 by microbial antigens enhances the NF-κB-mediated expression of DEFB4 in humans. Interestingly, the gene encoding NOD2 harbors at least two VDREs; as such, $1,25(OH)_2D$ has been shown to strongly upregulate the expression of NOD2 in human myeloid and epithelial cells, thus indirectly stimulating DEFB4 induction [40]. Hepcidin – a protein known to modulate tissue distribution of iron via suppressing ferroportin-mediated export of intracellular iron – is another VitD-responsive AMP. Studies show that calcitriol inhibits the expression of hepcidin in hepatocytes and monocytes, thereby facilitating export of intracellular iron and decreasing its intracellular concentrations. As iron is vital for bacterial survival and proliferation, this effect provides protection against intracellular pathogens [41].

Upon induction by TLR2/1 signaling, VitD can directly exert its stimulatory effect on the production of AMPs. However, it should be noted that this pathway can be differentially influenced by T-cell cytokines as well. Studies show that IL-15 and the Th1 cytokine, IFN-γ, synergize with TLR2/1 ligands in inducing CYP27B1 activity and thus enhance the induction of CAMP and DEFB4 expression in macrophages, whereas the Th2 cytokine, IL-4, promotes the activity of CYP24A1, which catalyzes vitamin D to an inactive metabolite, and strongly suppresses the vitamin D-mediated induction of CAMP and DEFB4 [42]. These findings suggest a link between innate and adaptive immune responses through the immunomodulatory function of VitD, although the exact implications of this are not yet understood.

Interestingly, although TLR activation upregulates the production of calcitriol through inducing CYP28B1, calcitriol has been shown to inhibit the expression of TLRs in a time- and dose-dependent fashion, thus forming a classic negative feedback mechanism [43]. Regulation of TLR expression by $1,25(OH)_2D$ may be mediated through downregulation of miR155, which subsequently stimulates SOCS1 [44]. Moreover, calcitriol upregulates the expression of CYP24A1, the calcitriol inactivating enzyme. This phenomenon leads into decreased responsiveness to pathogen-induced molecular cascades, thus self-inhibiting excessive TLR activation and unresolved inflammation and tissue damage at further stages of infection [45].

There are other instances of VitD acting as an inducer of immunotolerance in the innate immune system. Although it is known that $1,25(OH)_2D$ stimulates the differentiation of monocytes into mature macrophages [46], studies suggest that calcitriol favors the polarization of macrophages into an anti-inflammatory M2 phenotype. The M2 phenotype is associated with the production of anti-inflammatory cytokines such as IL-10, while M1 macrophages tend to propagate the inflammatory cascade through upregulation of pro-inflammatory mediators and promote Th1 and Th17 adaptive immune responses, aiming to recruit additional inflammatory cell types to the site of inflammation [47, 48]. Studies show that calcitriol suppresses the production of inflammatory mediators such as nitric oxide, TNF-α, IL-23, IL-12, IL-6, RANKL, COX-2, and IL-1β by macrophages [48]. Current evidence indicates that calcitriol exerts its anti-inflammatory effect on macrophages through downregulation of intracellular inflammatory signaling pathways (e.g., NF-κB and mitogen-activated protein kinase (MAPK) pathways) that are activated through TLR signaling upon pathogen recognition and are responsible for transcription of pro-inflammatory cytokines and perpetuation of the inflammatory cascade. IκB-α is an inhibitory protein which suppresses NF-κB signaling via attaching to NF-κB subunits and preventing its nuclear translocation. Incubation of LPS-stimulated macrophages, as well as respiratory epithelial cells with $1,25(OH)_2D$, has been shown to upregulate IκB-α levels, thereby inhibiting NF-κB signaling and its associated pro-

inflammatory cytokines [49]. Similarly, the production of MKP-1 – an inhibitor of the MAPK pathway – has been shown to increase in response to calcitriol treatment of monocytes [48].

The regulatory role of calcitriol in the production of AMPs is not limited to the classical immune cell types and is common to many human tissues, thus protecting against a wide range of disease scenarios. Current evidence strongly supports upregulated expression of AMPs in respiratory and intestinal epithelial cells, keratinocytes, uroepithelium, placental trophoblasts, and decidual cells in response to both endocrine and TLR-induced autocrine $1,25(OH)_2D$ pathways; through which VitD contributes to the innate host response against pathogens at mucosal surfaces of the body [50–54]. In keratinocytes, calcitriol was shown to enhance TLR2/1 and CAMP expression, thus resulting in increased antimicrobial activity against *Staphylococcus aureus* [55]. Intestinal epithelial cells are constantly exposed to luminal bacteria and play a key role in innate immunity. Paneth cells – intestinal epithelial cells known to secrete antimicrobial peptides – have been found to be regulated by VDR. Moreover, studies in VDR-null mice demonstrated increased intestinal bacterial loads [52, 56]. $1,25(OH)_2D$-mediated induction of CAMP expression in both placental trophoblasts and bronchial epithelial cells independent from TLR signaling has been observed, indicating a pivotal role for VitD in protection against infections during pregnancy and respiratory infections, respectively [51, 54]. Induction of cathelicidin production upon VitD treatment has been shown to enhance antibacterial activity against *Pseudomonas aeruginosa* and *Bordetella bronchiseptica* in bronchial epithelial cells of cystic fibrosis patients [54]. Interestingly, in biliary epithelial cells, expression of CAMP is regulated by bile acids through the VDR, indicating that VDR can function as a bile acid sensor [57, 58]. In addition to VitD-mediated upregulation of AMPs in epithelial cells throughout the body, the expression of VDR and CYP27B1 by epithelial cells suggests that VitD might also play a crucial role in physical barrier component of innate immunity through regulating epithelial intracellular functions. This notion is supported by the finding that calcitriol maintains barrier integrity through upregulating the expression of epithelial junctional proteins such as tight junctions (e.g., occludin), gap junctions (e.g., connexin 43), and adherens junctions (e.g., E-cadherin) [59, 60].

Effects of calcitriol on the innate immune system extend beyond regulation of AMPs. $1,25(OH)_2D$ has been found to promote proliferation of monocytes and their differentiation into mature macrophages [46]. Maturation of phagosomes is enhanced by VitD, leading into an improved capacity for phagocytosis and autophagy [61]. Autophagy is the process of degrading intracellular engulfed material through phagolysosomal fusion and has been implicated as a mechanism enhancing antigen presentation in viral and bacterial infections. The strong induction of autophagy by VitD is of particular importance since it suggests that VitD might facilitate antigen presentation by monocytes and macrophages through inducing autophagy [62, 63]. VitD also stimulates chemoattraction of neutrophils and monocytes and induces production of the lysosomal enzyme acid phosphatase as well as reactive oxygen intermediates such as hydrogen peroxide [64, 65]. Aberrant maturation, phagocytosis, chemotaxis, and cytokine production have been detected in monocytes and macrophages of VitD-deficient subjects [66]. Finally, $1,25(OH)_2D$ has been found to strongly induce the expression of CD14, a TLR co-receptor critical for recognition of LPS by innate immune cells [39]. This effect provides the defense cells with the ability to rapidly sense and respond to pathogen-associated TLR ligands.

As cathelicidin is known to display antiviral effects, it is reasonable to assume that VitD is involved in host defense against viral infections as well. Studies have reported VitD-mediated inhibition of HIV replication in macrophages via induction of cathelicidin, possibly through enhanced autophagy and phagosomal maturation [67]. Cathelicidin induction by VitD may also enhance protection against influenza [68]. VitD-mediated induction of CAMP has been observed in lung epithelial cells following viral infection [69].

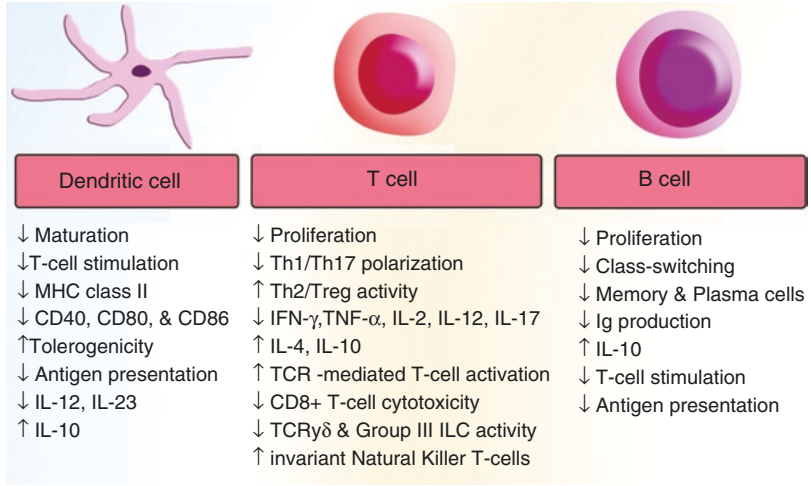

Fig. 2.3 VitD in adaptive immunity. MHC major histocompatibility complex, IL interleukin, Th T-helper, Treg regulatory T cell, IFN interferon, TNF tumor necrosis factor, TCR T-cell receptor, ILC innate lymphoid cell, Ig immunoglobulin

The discussed studies highlight the crucial function of VitD as a stimulant of innate immunity with broad-reaching antimicrobial effects on several immune and immune-related cell types. In light of these findings, and considering the growing prevalence of antibiotic-resistant infections, strategies that are able to boost antimicrobial effects of VitD represent novel approaches of improving innate immunity to infection. Histone deacetylase inhibitors such as butyrate have been observed to enhance VitD-mediated induction of cathelicidin production [70] and therefore are promising candidates for treating infections.

VitD and Dendritic Cells

Dendritic cells (DCs) are the bridge between the innate and adaptive arms of the immune response. DCs are the most potent antigen-presenting cell (APC) within the immune system. They intercept and process foreign antigens and present them as peptides to T and B cells. Through spreading immunogenic or tolerogenic signals, DCs program the polarization and differentiation of lymphocytes into adequate effector cell types and thus initiate and modulate the adaptive immune response.

DCs are important targets for immunoregulatory effects of VitD (Fig. 2.3). They express both VDR and CYP27B1 and therefore can accumulate relevant local concentrations of $1,25(OH)_2D$. Current evidence strongly suggests that calcitriol promotes immune tolerance in the adaptive immune system via alteration of DC function and morphology to a tolerogenic, immature state [71, 72]. Both 25(OH)D and $1,25(OH)_2D$ are shown to block maturation and immunostimulatory capacity of DCs and preserve a hyporesponsive tolerogenic DC phenotype characterized by reduced expression of antigen-presenting molecules MHC class II as well as co-stimulatory molecules (e.g., CD40, CD80, CD86). Tolerogenic DCs are relatively resistant to maturation, manifest reduced antigen presentation activity, and are poor inducers of $CD4^+$ T-cell function [73–76]. In contrast, they enhance the activity of regulatory T cells (Tregs) [77], which are critical for controlling the immune response and mediating immune tolerance. Moreover, tolerogenic DCs also stimulate apoptosis of autoreactive T cells [78].

Interestingly, DCs express higher levels of CYP27B1 and lower levels of VDR during maturation into APCs [79, 80]. In view of suppressive effect of calcitriol on DC function, both these effects are thought to prevent from overstimulation of mature DCs and potential aberrant immune responses. Moreover, the paradox between upregulation of calcitriol production and downregulation of VDR expression in mature DCs has been speculated to indicate that the calcitriol synthesized by mature DCs is utilized in paracrine regulation of immature VDR-expressing DCs.

The tolerogenic effect of calcitriol on DCs was confirmed when it was shown that VDR and CYP27B1 knockout mice present with significantly increased numbers of mature DCs and manifest lymphatic abnormalities consistent with abnormal DC trafficking [81]. Moreover, treatment of DCs with calcitriol was found to repress the secretion of pro-inflammatory cytokines such as IL-12 and TNF-α, which are known to drive Th1/Th17 T-cell responses. Instead, it increased the production of the tolerogenic Treg-promoting cytokine, IL-10 [72, 73]. Inhibition of IL-12 production by calcitriol is not only because of the explained altered differentiation of DCs but also arises from the direct effect of calcitriol on transcription of IL-12. The $1,25(OH)_2$D-VDR-RXR complex is known to bind to specific binding sites of NF-κB in the promoter of IL-12 gene and thereby prevents the NF-κB-mediated activation of IL-12 transcription [75]. $1,25(OH)_2$D was shown to inhibit differentiation of monocytes into DCs in vitro [76].

The VitD-induced inhibition of DC maturation and promotion of tolerogenic DC response has introduced the concept that low VitD status might be associated with an increased risk for autoimmunity. It is known that antigen presentation by immature DCs facilitates immune tolerance, while antigen presentation by mature DCs conveys more immunogenicity. In the normal state, immature DCs are predominantly responsible for the presentation of self-antigens so as to maintain self-tolerance. The role of VitD in autoimmunity is further discussed in the sections that follow.

VitD and Adaptive Immunity

T Cells

T cells are traditionally divided into distinct subpopulations. $CD4^+$ T-helper (Th) cells are responsible for regulation of T- and B-cell responses. Under the influence of APCs and many other immunomodulators, naïve Th cells are polarized into functionally distinct subsets including Th1, Th2, and Th17 cells. Each subset initiates a distinct pattern of immune response through secretion of a specific profile of inflammatory cytokines. $CD8^+$ cytotoxic T cells are responsible for direct cellular defense against target cells including tumoral and virus-infected cells. The regulatory Th cells (Tregs) are part of the machinery responsible for maintenance of immune self-tolerance and are crucial for controlling overexuberant immune responses through down-regulating the activity of macrophages, DCs, $CD4^+$, and $CD8^+$ T cells and producing anti-inflammatory cytokines.

T cells express the 1-α hydroxylase enzyme CYP27B1 and upregulate CYP27B1 expression upon activation. Although resting memory and naïve T cells express very low levels of VDR, expression of VDR is remarkably upregulated upon activation of the T-cell receptor (TCR) signaling and correlates with the level of T-cell stimulation [82, 83]. VitD is a potent modulator of adaptive immune response and has a part in shaping B- and T-cell immune responses (Fig. 2.3). It has been suggested that VitD affects the function of T cells through four potential mechanisms: 1) endocrine effects mediated by circulating calcitriol synthesized by the kidneys, 2) autocrine effects mediated by calcitriol synthesized by T cells, 3) paracrine effects mediated by calcitriol synthesized by the neighboring monocytes and DCs, and 4) indirect modulation of T-cell differentiation and function via regulation of DCs.

The influence of calcitriol on differential activation and polarization of Th subsets has been extensively studied, and Th cells appear to be the principal target for VitD. VitD inhibits Th cell proliferation and differentiation and modulates their cytokine production pattern [84]. Both antigen- and IL-2-induced proliferation of $CD4^+$ and $CD8^+$ memory T cells have been shown to be directly inhibited by $1,25(OH)_2$D [85]. Calcitriol is thought to exert its inhibitory effects on the $CD4^+$ T-cell response through suppression of both DC maturation and antigen presentation, and also direct effects on the VDR of T cells. However, the extent of the contribution of each mechanism is not yet clear. $1,25(OH)_2$D signaling is known to diminish the Th1 response, as evidenced by VitD-mediated inhibition of key Th1 pro-inflammatory cytokines such as IFN-γ,

TNF-α, IL-2, IL-6, IL-8, IL-9, IL-12, and IL-22. In contrast, calcitriol stimulates Th2 response and upregulates secretion of Th2-associated cytokines such as IL-3, IL-4, IL-5, and IL-10 [86–89]. As excessive skewing of the Th response toward the Th1 phenotype has been implicated in pathogenesis of autoimmunity, effects of VitD on the Th subsets are thought to maintain the Th1/Th2 balance and prevent from aberrant autoimmune responses.

Evidence links activation of IL-17-producing Th17 cells to the pathogenesis of autoimmune disorders [90]. Activation of Th17 cells and overexpression of IL-17 have been found to play a key role in mediating murine models of autoimmune diseases including experimental autoimmune encephalitis (EAE) and inflammatory arthritis, as well as human rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) [91–94]. $1,25(OH)_2D$ directly suppresses the production of IL-17 on a transcriptional level [94]. Calcitriol treatment of activated human T cells results in significantly reduced levels of IL-17, IFN-γ, and IL-21 production [95]. Decreased IL-17 levels have also been reported in autoimmune disease-susceptible nonobese diabetic (NOD) mice following VitD treatment [96]. $CD4^+$ T cells of VDR-knockout mice secrete higher levels of IFN-γ and IL-17 compared with those of wild-type mice [86]. CYP27B1-knockout mice have also been detected with increased levels of IL-17 production in proximal and distal colon, which was associated with weight loss and colitis [52]. Calcitriol represses differentiation and activation of Th17 cells through suppression of Th17-related cytokines and transcription factors such as IL-17A, IL17F, IL-21, RORC, and CCR6 [88, 97]. $1,25(OH)_2D$-exposed Th17 cells are less likely to activate synovial fibroblasts and to mediate EAE [94, 98], and VitD treatment has been found to suppress murine retinal autoimmunity following decreased Th17 activity [99].

Although the exact mechanisms involved in regulation of Th1 and Th2 immune responses by VitD are yet not clear, available evidence indicates direct calcitriol/VDR-driven effects at a transcriptional level. Studies have reported direct calcitriol-induced inhibition of IL-2 transcription through blocking NFAT/AP-1 complex formation via binding of the calcitriol-VDR-RXR complex to the NFAT element in the IL-2 promoter [100]. Upregulation of the NF-κB inhibitory protein IκB-α and the Th2-promoting transcription factor GATA3 following $1,25(OH)_2D$ treatment have also been documented [7, 101]. Direct binding of calcitriol-VDR-RXR complex to a silencer VDRE in the promoter of IFN-γ gene has been suggested as the potential mechanism of suppression of IFN-γ secretion by calcitriol [102]. Regarding inhibition of IL-17 production, several mechanisms have been proposed, including blocking NFAT and Runx1 binding to the IL-17 promoter possibly via induction of Foxp3, inhibiting the Th17-polarizing transcription factor RORνt, and inhibiting Smad7 transcription [76, 94, 103].

Tregs are the tolerogenic subset of $CD4^+$ T cells characterized by expression of the inhibitory co-receptor CTLA4 and regulated by the transcription factor FoxP3. The function of Tregs is critical for prevention of autoreactivity and exaggerated immune responses. Subjects with FoxP3 mutations suffer from the IPEX syndrome characterized by a plethora of autoimmune disorders [104]. We previously noted that, through induction of tolerogenic DCs, VitD stimulates the function of Tregs. However, VitD is also known to directly stimulate the differentiation and activation of Tregs at a transcriptional level. The FoxP3 gene promoter region is known to harbor at least one VDRE, and calcitriol directly upregulates FoxP3 expression [105]. Various studies have confirmed that both 25(OH)D and $1,25(OH)_2D$ enhance generation of $CTLA4^+$ and $FoxP3^+$ IL-10-secreting Tregs [95, 106]. Adding a combination of calcitriol and IL-2 to human primary T-cell cultures resulted in promoted expression of genes characteristic for Tregs. In mice treated with either $1,25(OH)_2D$ or UVB radiation, Tregs that originated from draining lymph nodes were more effective in suppressing antigen-specific immune responses and production of autoantibodies upon adoptive transfer into untreated mice [107, 108]. Furthermore, calcitriol augments expression of indoleamine 2,3-dioxygen-

ase (IDO) enzyme, which is known to expand the Treg population [109].

Our current understanding of the role of VitD in modulation of T-cell functions mostly stems from mechanistic studies that have focused primarily on the response of these cells to $1,25(OH)_2D$ treatment in vitro. However, how variations in VitD status affect the function of different T-cell subsets is less clear. There are a few reports linking VitD serum levels with specific T-cell subpopulations. For instance, 25(OH)D serum levels have been shown to correlate with Treg immunosuppressive capacity in patients with multiple sclerosis [110]. Furthermore, VitD supplementation was shown to significantly increase circulating Treg cell numbers in both renal transplant recipients and healthy subjects [111, 112].

Studies conducted in VDR-knockout mice have also provided some insight into the role of VitD in T-cell immune response. It is known that calcitriol suppresses T-cell proliferation and restricts Th1/Th17 differentiation. These effects might promote immune tolerance and prevent autoimmunity, which is in line with clinical findings that have correlated VitD deficiency with a higher incidence of autoimmune disorders. However, as Th1/Th17 responses are important to mount effective immune response during infections, calcitriol would be expected to have detrimental effects on host defense against certain pathogens. However, VDR-knockout mice did not manifest decreased or increased susceptibility to infections that require Th1/Th17-mediated immune response, including *Listeria monocytogenes, Leishmania major, Mycobacterium bovis, Mycobacterium tuberculosis, Candida albicans, Herpes simplex, Schistosoma mansoni,* and *Bordetella pertussis* [113]. More strikingly, human epidemiologic studies have linked VitD deficiency with higher risk of infection. Although there is still no generally approved explanation for this paradox, the contradictory effect of VitD on innate and adaptive immune responses is a possible explanation. Calcitriol strengthens the innate host defense while modulating the adaptive response to a more tolerant state to limit excessive inflammation. Moreover, increased susceptibility to infection in VitD deficiency could be explained by the recent findings describing the crucial role of calcitriol in TCR-mediated activation of naïve T cells. During the initiation of naïve T-cell response, engagement of TCR by the antigen results in p38 MAPK-dependent stimulation of VDR, which is required for induction of phospholipase C-γ1 (PLC-γ1). PLC-γ1 is a cofactor of the classical TCR signaling pathway and essential to subsequent TCR signaling and full T-cell activation [114]. The expression level of VDR in naïve $CD4^+$ is shown to correlate with the degree of T-cell activation [115], although some have proposed that calcitriol might affect T-cell activation via direct modulation of the TCR [116]. Additionally, VitD might play a role in promotion of lymphocyte migration and trafficking, as 25(OH)D and $1,25(OH)_2D$ treatment of naïve and effector T cells has been found to upregulate expression of CCR10 and CCR5 in vitro [117].

It is interesting to note that in line with all other effects of VitD on the function of T cells, regulation of TCR-mediated T-cell activation by VitD is also tailored to minimize the risk of overstimulation of the T-cell-driven immune response. Studies show that there is an approximate 48-hour delay between initial TCR stimulation by the antigens and full induction of PLC-γ1 by calcitriol. In case the innate host defense manages to adequately control the infection during this lag period, then limited concentrations of antigen would coincide with the onset of T-cell proliferatory response afterward, thus providing a relatively uninflammatory microenvironment and preventing from an explosive acquired immune response. However, in case the innate response is inadequate in controlling the invading pathogens during the lag period, then elevated concentrations of antigen will boost a more aggressive adaptive response [95].

Besides $CD4^+$ Th cells, calcitriol seems to modulate the function of several other T-cell subpopulations as well. Compared to $CD4^+$ T cells, $CD8^+$ T cells express even higher levels of VDR. Calcitriol suppresses the generation and activity of $CD8^+$ T cells and production of IFN-γ

and TNF-α by these cells [118]. VDR-null mice exhibit increased numbers of $CD8^+$ T cells [118]. Furthermore, calcitriol inhibits IFN-γ secretion by unconventional TCRγδ T cells [119]. Both $CD8^+$ T cells and TCRγδ T cells have been implicated to play a role in autoimmune disorders including multiple sclerosis (MS), inflammatory bowel disease (IBD), and psoriatic arthritis [120]. It has also been suggested that VitD contributes to maturation and function of invariant natural killer T cells (iNKTs). as VDR-knockout mice are found to develop functionally immature iNKTs that are hyporesponsive to TCR stimulation [121]. Finally, increased levels of group 3 innate lymphoid cells – which has been reported in various autoimmune conditions including psoriasis, Crohn's disease, and MS – have been detected in VDR-knockout mice [89, 122].

Taken together, VitD shifts the adaptive immune response from a pro-inflammatory to a more tolerogenic status, through maintaining the Th1/Th2 balance, downregulating the Th17 immune response, stimulating Treg activity, and modulating TCR signaling. This effect is consistent with the tolerogenic effect of calcitriol on the DCs.

B Cells

In line with its role in modulation of DCs and T-cell response, VitD suppresses the function of B lymphocytes and favors a self-tolerant B-cell response. B cells constitutively express CYP28B1 and VDR and upregulate both upon activation. VitD affects several aspects of B-cell homeostasis both indirectly via modulation of the T-helper immune response and directly via intracrine effects in the VDR-expressing B cells.

Calcitriol directly inhibits ongoing proliferation of B cells. As a result, VitD suppresses the generation of class-switched memory B cells from naïve B cells [123] and differentiation of B cells into plasma cells through directly blocking NF-κB activity downstream to CD40 activation [124]. Apoptosis of immunoglobulin-producing B cells is enhanced through direct effect of calcitriol [125]. VitD directly inhibits immunoglobulin class switching and production of antibodies by B cells [126]. VDR-null mice exhibit enhanced levels of IgE production [127]. These findings are clinically important as they lend support for the potential role of VitD deficiency in pathogenesis of antibody-mediated autoimmune disorders as well as various B-cell-associated disease conditions such as IgE-mediated asthma and other allergic disorders.

Regulation of other immune cells via secretion of cytokines and expression of surface proteins is another contribution of B cells to the immune system. Calcitriol enhances secretion of the anti-inflammatory cytokine IL-10 by B cells as it directly binds to a silencer VDRE in IL-10 gene promoter [128], thus suggesting a protective role for VitD in allergic immune responses. VitD downregulates the expression of CD86 on B cells, which results in reduced stimulation of T cells [129]. B cells can also act as APCs. $1,25(OH)_2D$ is shown to repress the expression of CD74 on B cells, which subsequently inhibits the assembly and surface exposure of MHC-II molecules [130].

VitD Status and Disease

As summarized in the preceding section, VitD is a key regulator of immune functions, with widespread influence on both innate and adaptive immunity. VitD potentiates the innate immune response not only in classic immune and inflammatory cell types but also in a variety of immune-related tissues throughout the body. Calcitriol also modulates the adaptive response toward a more self-tolerant and balanced phenotype. These effects are mediated by the circulating calcitriol, as well as the calcitriol synthesized by CYP27B1-expressing immune cells and their neighboring cells from circulating 25(OH)D, acting via autocrine and paracrine mechanisms. Normal effects of VitD on target cells require a threshold level of VDR engagement by $1,25(OH)_2D$ and thereby depend on availability of sufficient levels of circulating 25(OH)D and $1,25(OH)_2D$. Based on these facts, it could be speculated that VitD status of an individual affects VitD-dependent immune functions; in other words, VitD-deficient subjects might be prone to various immune-related

pathologic conditions including autoimmune, inflammatory, and infectious disorders. In this section, we highlight examples of human studies that aimed to explore the role of VitD status in selected diseases. These studies can be categorized into two groups. First are epidemiologic studies that try to correlate VitD deficiency with prevalence or incidence of certain disease states. Second are interventional studies that have investigated the effect of VitD supplementation on incidence or severity of diseases.

Before embarking on this part of the chapter, it is important to recognize that clinical investigation of the role of VitD in human health and disease is not without challenges and limitations. First, it should be noted that clinical extrapolation of the mechanistic evidence outlined above, which in part derives from study of animal models, should be performed with caution, as involvement of VitD in regulation of many immune pathways is species-specific. For instance, the array of genes encoding AMPs and their VDREs, as well as cytokines and chemokines, and TLRs involved in innate immune system varies greatly among species [131, 132]. Moreover, when interpreting data from in vitro studies using cell cultures, it should be kept in mind that these studies involve the use of *exogenous* calcitriol in higher concentrations than physiological circulating range.

Moreover, many clinical studies that have addressed the role of VitD status in disease states are of cross-sectional or retrospective design. Another concern is to abstain from establishing causal relationships based largely (or entirely) on the results of these "association" studies. VitD deficiency and impaired extrarenal activity of VitD have been described as a consequence of several pathologic conditions, therefore detecting an association between VitD deficiency and prevalence or severity of a disease does not necessarily provide evidence for the role of VitD deficiency as an etiologic factor. However, the growing number of prospective controlled studies (e.g., large cohort studies and interventional trials) have shed new light on our understanding of this association.

While one would hope that randomized controlled trials (RCTs) on the actual effects of VitD administration on disease incidence or severity parameters would provide solid answers, they have produced mixed and sometimes conflicting results. Diverse reasons might have contributed to the negative outcomes. In many cases, RCTs with negative results have either not documented the baseline VitD status of the treated population or the effectiveness of VitD supplementation regimen in raising VitD levels. Thus, these studies failed to direct the intervention to subgroups of participants who might actually benefit from treatment; the possible inclusion of already VitD-replete subjects would weaken the overall treatment effect. However, in this regard, there is a discrepancy in definition of VitD deficiency or sufficiency thresholds between various medical organizations. In addition to the available VitD status classifications being inconsistent, the groupings are based on bone health endpoints (especially bone mineral density) and ignore extra-skeletal tissue-specific VitD requirements. For example, it has been shown that up to the 75 nmol/l (30 ng/ml) concentration, the serum 25(OH)D levels correlate with serum PTH levels as well as intestinal calcium uptake, thus suggesting this threshold as a potentially relevant indicator of sufficient VitD status in regard with bone and mineral homeostasis [133, 134]. However, a certain level of VitD that is deemed to provide normal bone and mineral homeostasis does not necessarily fully satisfy the physiologic needs of other VitD-dependent body systems, including the immune system. Therefore, the target VitD level that is required to maintain optimal VitD physiological actions in all aspects of human health is unascertained and should be clearly defined and validated through well-designed studies examining non-bone endpoints. Otherwise, it will not be feasible to determine the optimal dosage, frequency, duration, and mode of supplementation for various disease conditions and different severities of deficiency. However, the fact that multiple individual factors such as lifestyle, clothing, skin health, skin pigmentation, and age contribute to the amount of endogenous VitD synthesis and subsequently circulating 25(OH)D levels further complicates the possibility of tailoring general recommendations for VitD supplementation without the use of complex risk stratification tools.

VitD supplements are easily accessible over the counter. VitD-fortified products are also available in many countries. Moreover, VitD is synthesized in the skin constantly albeit in variable quantities according to season, clothing, lifestyle, etc. Natural dietary sources of VitD, although limited, are also at hand. Therefore, another issue that complicates clinical trials of VitD is the possibility of improvements in VitD levels of the control group, which could mask the treatment effects.

Virtually all human studies have used the serum concentrations of the precursor 25(OH)D as the determinant of VitD status of an individual. However, it is known that the active metabolite $1,25(OH)_2D$ is responsible for physiologic functions of VitD. Calcitriol is available in minute amounts in circulation and has a short half-life. Moreover, immunomodulatory effects of VitD are largely dependent on autocrine and paracrine pathways of calcitriol synthesis and metabolism, which are localized to the target tissues. Given the complexity of VitD metabolism (Fig. 2.1), which involves endocrine as well as intracrine and paracrine regulation, some argue that circulating 25(OH)D concentrations may not adequately reflect local tissue VitD availability. One potential future perspective is the measurement of concentrations of "free" or non-DBP-bound (i.e., free plus albumin-bound) VitD metabolites in addition to total serum levels, as target cells seem to be more responsive to the non-DBP-bound metabolites. This strategy has already been corroborated in studies of skeletal effects of VitD in healthy subjects, where free 25(OH)D levels were shown to better correlate with bone mineral density as compared to total 25(OH)D concentrations [135]. While free VitD levels can be estimated using total 25(OH)D, DBP, and albumin levels, novel laboratory techniques are making direct measurement of free 25(OH)D levels feasible as well. Moreover, as will be discussed later in the chapter, recent studies have introduced the concept that there are factors besides serum 25(OH)D levels that might influence the extent of VitD biological effects. For instance, genetic polymorphisms in the proteins, enzymes, and receptors involved in VitD metabolism have been shown to affect VitD's functional bioavailability. In conclusion, the use of novel indicators of VitD status which can reflect the actual end-organ bioavailability of calcitriol at the target tissues can result in more accurate identification of VitD-deficient subjects to whom the therapeutic intervention should be directed and provide an enhanced prediction of treatment response. Ideally, these future biomarkers should incorporate inherited genetic variations that affect VitD functionality irrespective of serum 25(OH)D levels, thus introducing the concept of patient-specific target VitD status, consistent with the goals of personalized medicine. In this regard, large-scale microarray analysis and ChiP sequencing studies are in development [136, 137].

Autoimmune Conditions

As described above, VitD is a tolerogenic immunomodulator and regulates several immune cell types involved in prevention or propagation of autoimmunity. It suppresses maturation and antigen presentation of DCs and promotes the development of tolerogenic DCs. VitD suppresses/diminishes both Th1 and Th17 responses, both strongly indicated in the pathogenesis of autoimmunity. It also stimulates activity of Tregs and coordinates TCR activation of naïve T cells to prevent an overexuberant T-cell response. VitD also suppresses $CD8^+$ T cells and inhibits the production of autoantibodies by class-switched B cells. Moreover, although an inducer of innate immunity, calcitriol inhibits expression of TLRs through a negative feedback mechanism and represses the production of pro-inflammatory cytokines by monocytes and macrophages, thus preventing from an exaggerated innate defense. Collectively, these findings strongly implicate that sufficient 25(OH)D levels are essential for preserving normal immune homeostasis and VitD deficiency might contribute to development of autoimmune disorders in genetically susceptible individuals. VitD deficiency is highly common among patients with autoimmune disorders [21]. Interestingly, VDREs have been identified in

close proximity to several single nucleotide polymorphisms (SNPs) that have been associated with various autoimmune diseases [138]. Many studies have addressed the potential role of VitD deficiency in major autoimmune conditions.

Type 1 Diabetes Mellitus (T1DM)

T1DM is mainly characterized by infiltration of cytotoxic $CD8^+$ T cells into the pancreatic Langerhans islets and associated destruction of the insulin-producing β cells. This is an IL-12-dependent process regulated by the Th1 response. We previously noted that VitD suppresses both Th1 and $CD8^+$ cells. Treatment of nonobese diabetic (NOD) mice – an animal model for human T1DM – with a calcitriol analog suppressed pancreatic infiltration of Th1 cells and production of IL-12 while expanding the population of Tregs in pancreatic lymph nodes [139, 140]. VitD-deficient mice, in contrast to VitD-supplemented mice, demonstrate earlier onset and higher occurrence of insulitis and diabetes in early life [141, 142].

Observational data suggest a correlation between VitD deficiency and T1DM [143, 144]. Studies have described a seasonal pattern for incidence of T1DM, which has been found to be higher during winter compared with summer, implying the potential effect of sunlight exposure and hence the protective role of endogenous synthesis of VitD [145, 146]. Compared to healthy individuals, serum 25(OH)D levels were significantly lower in T1DM patients at the time of diagnosis [144]. Incidence of T1DM was found to be three times higher in children with suspected rickets, compared to VitD-sufficient controls [147].

Studies of VitD supplementation provide results in favor of a protective effect of VitD against the development of T1DM in children. Two case-control studies revealed that vitamin D supplementation or cod liver oil intake during infancy significantly decreased the risk of developing T1DM [148, 149]. Two birth cohort studies also reported that VitD supplementation during the first year of life could reduce the incidence of T1DM by 33% and 80% [147, 148]. A meta-analysis of four large trials confirmed a significantly reduced risk of T1DM development in VitD supplemented infants (pooled odds ratio = 0.71) [150]. VitD treatment of pregnant women whose offspring were at risk of developing T1DM decreased the risk of developing islet autoantibodies in their children, thus marking the effect of in utero exposure to VitD on incidence of pancreatic autoimmunity [151]. However, VitD supplementation studies in adults have produced mixed results. While two supplementation studies did not show any beneficial effects [152, 153], a randomized, double-blind, placebo-controlled clinical trial recently reported recovery of β-cell function following supplementation of 38 T1DM patients with 2000 IU of cholecalciferol for 18 months [154]. These inconsistent results might arise from the relative irreversibility of β-cell destruction. A protective effect of VitD in adult T1DM patients was only detected when the disease duration was less than 1 year [155]. In conjunction with the promising results in children, current studies suggest that VitD supplementation is most beneficial when administered early in the disease pathogenesis.

Systemic Lupus Erythematosus (SLE)

SLE is an antibody-mediated autoimmune disease. As discussed above, VitD suppresses differentiation and autoantibody production by B cells. Calcitriol treatment has been shown to reduce the severity of SLE in MRL/1 mice [156]. B cells extracted from active SLE patients manifest a significantly reduced spontaneous and stimulated polyclonal antibody production as well as up to 60% reduction in spontaneous anti-dsDNA autoantibody production when preincubated with calcitriol [157]. Interferon signature – i.e., the characteristic overexpression of IFNα-inducible genes in peripheral blood mononuclear cells – is observed in about 50% of SLE patients and correlates with disease severity [158, 159]. In SLE subjects, VitD deficiency is shown to be associated with the interferon signature, the risk of which can be lowered by 2.1-fold through VitD supplementation [160].

Cross-sectional studies have reported lower 25(OH)D levels in SLE patients in comparison with the normal population [161–163]. Nevertheless, VitD supplementation studies have

reported inconclusive results. A prospective study failed to detect any difference in the incidence of SLE between VitD-supplemented and control groups [164]. In healthy subjects, VitD deficiency was correlated with the presence of lupus autoantibodies, the concentrations of which decreased following VitD supplementation [165].

Another group of studies have evaluated the association between VitD levels and disease activity in SLE patients and have again obtained mixed results. While some have failed to correlate VitD deficiency with SLE flare-up [166, 167], others have found an inverse correlation between serum 25(OH)D levels and disease activity, proteinuria, and pro-inflammatory cytokine production [168, 169]. For instance, in children with juvenile SLE, lower serum 25(OH)D levels were associated with a higher disease activity [170]. An important interventional study reported improved disease activity score and fatigue as well as decreased autoantibody levels in 158 VitD-treated SLE patients compared with 89 placebo-treated SLE subjects [171].

In summary, although most epidemiologic studies have linked VitD deficiency with higher SLE prevalence or disease activity, a causal relationship between VitD status and SLE incidence or severity could not be established on this basis. For example, hypovitaminosis D might be a consequence of SLE patients' avoidance of sunlight exposure due to their photosensitivity. On the other hand, prospective controlled evidence currently available is both limited and inconclusive; hence, more well-designed studies are needed to settle this controversy.

Multiple Sclerosis (MS)

MS is an autoimmune disease involving T-cell-mediated inflammation of the central nervous system (CNS). VitD treatment of murine models of MS (EAE) has been effective in prevention of disease onset and reversal of paralysis in animals with ongoing disease [172]. Calcitriol treatment prevents $CD4^+$ T-cell proliferation and migration into the CNS and leads into decreased number of active Th17 cells within the CNS and their downregulated production of IL-17 and IFN-γ [173]. As mentioned earlier, human 25(OH)D levels directly correlate with Treg numbers and activity. Through a negative feedback mechanism, calcitriol reduces pro-inflammatory cytokine production in brain pericytes, thus preventing excessive neural inflammation [174]. Recent studies have detected upregulated expression of VDR and CYP27B1 within the chronic active MS brain lesions compared to healthy brain tissue, suggesting a potential endogenous role for VitD in suppression of active MS lesions [175].

Epidemiological evidence strongly supports a link between VitD deficiency and development of MS [176, 177]. Subjects living at latitudes below 35° during the first 10 years of life reveal a 50% reduction in the risk of MS [178]. Risk of MS decreases by 41% for every 20 ng/ml increase of 25(OH)D levels above 24 ng/ml [176]. Women with high VitD intakes are 40% less likely to develop MS [179]. Furthermore, MS patients who are in remission have significantly higher VitD levels compared to relapsed patients [180]. Magnetic resonance imaging (MRI)-determined MS disease activity was lower in VitD-sufficient MS patients [181]. IFN-β therapy of VitD-sufficient MS patients is associated with a lower relapse rate compared to that of VitD-deficient patients [182].

Similar to other autoimmune disorders, VitD supplementation studies in MS have produced mixed results. VitD supplementation with more than 400 IU per day decreased the risk of developing MS by 42% [179]. Cholecalciferol supplementation of MS patients improved the Expanded Disability Status Scale (EDSS), reduced MRI lesions and relapse rate, and increased functionality [183, 184]. These effects were more pronounced when VitD was used as an add-on therapy to IFN-β [184]. Interestingly, VitD administration has also been shown to limit progression of pre-MS conditions like optic neuritis to MS [185]. However, some recent trials did not find any beneficial effect of VitD treatment on disease activity of MS subjects [186, 187]. Two recent randomized placebo-controlled trials reported that VitD supplementation had no beneficial effects on brain MRI lesions, relapse rates, EDSS, or MS functional composite [187, 188].

Chronic Inflammatory Disorders

Asthma

Asthma is a chronic inflammatory disease of the airways, characterized by bronchial infiltration and inflammation, airway hyperresponsiveness, and reversible airway obstruction. Multiple studies have elucidated the beneficial effects of calcitriol on the cell types and disease processes involved in pathogenesis of asthma. Inhaled and systemic corticosteroids represent the current first-line therapy for chronic asthma and asthma exacerbations, respectively. Broad immunosuppressive effects of steroids target both pro-inflammatory and anti-inflammatory immune components alike. Dexamethasone is known to impair the activity of Tregs and their production of the anti-inflammatory cytokine, IL-10, in steroid-resistant asthma patients. Coadministration of calcitriol and dexamethasone is shown to restore the Treg-mediated IL-10 response and reverse steroid resistance in $CD4^+$ T cells of steroid-resistant asthmatic patients [189]. We also previously noted that calcitriol inhibits pro-inflammatory cytokine production by innate immune cells and various nonimmune cell types. In asthma, studies have revealed that calcitriol inhibits cytokine production and migration of mast cells, neutrophils, and eosinophils and reduces production of cytokines, matrix metalloproteases, and mucus by airway smooth muscle cells, all leading into decreased airway hyperresponsiveness, inflammation, and remodeling [190–192]. Finally, as mentioned before, calcitriol upregulates the expression of MKP-1, a crucial inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway, which is implicated in the pathogenesis of steroid resistance in asthma [193].

Most epidemiologic studies suggest an association between VitD deficiency and either the development or severity of asthma. Low maternal VitD intake (and presumably low VitD status) during pregnancy was associated with wheezing in offspring [194]. VitD-deficient children are prone to increased risk for developing asthma-related illnesses (e.g., recurrent wheezing) and experience more severe symptoms, more frequent exacerbations, and reduced lung function [195], whereas higher serum 25(OH)D levels are associated with improved asthma control in VitD-sufficient children [196]. A recent meta-analysis of 16 birth cohort studies reported that maternal cord or peripheral blood 25(OH)D levels inversely correlate with risk of wheezing illnesses and possibly asthma in offspring [197]. Low 25(OH)D levels in adult asthmatic patients are associated with severe or uncontrolled asthma and a greater decline in lung function [198, 199] and constitute a significant predictor of all-cause mortality [200]. Nevertheless, many epidemiologic studies have reported no association between VitD status and asthma development and severity [201–203]. For instance, a nested case-control study including 584 adult new-onset asthma patients indicated that low VitD status was not associated with incident of asthma [204]. In other studies, low cord blood 25(OH)D level did not correlate with development of physician-diagnosed asthma in children by the age of 5 years, in either the United States [205] or New Zealand [206]; however, the latter study did find a strong inverse association between cord blood 25(OH)D level and risk of recurrent wheezing. These findings highlight the distinction between acute respiratory infections (with associated, nonspecific "wheezing") and the onset of actual asthma, a diagnosis that is generally not possible until the child reaches at least 5 years of age. Studies purporting to prevent "asthma" during infancy or early childhood are more likely preventing serial respiratory infections, as compared to actual asthma.

Regardless of asthma pathogenesis, VitD appears to have a salutary role among patients with asthma. Available evidence indicates that supplementation of VitD-deficient asthmatic patients has encouraging effects on improving disease control and ameliorating symptoms [207]. Several trials demonstrated reduced asthma symptoms and respiratory infections in VitD-treated asthmatic children with low 25(OH)D levels [208]. Two meta-analysis studies pooled RCTs of high-dose VitD in pediatric asthma patients and reported a significant reduction in asthma exacerbations (relative risk = 0.41 in both studies) after VitD therapy [209, 210]. However,

no significant effects on lung function or symptom scores were detected [210]. Moreover, although recent RCTs of VitD supplementation of pregnant women showed no statistically significant effect on incidence of wheezing episodes or early "asthma" in their offspring [211–213], VitD supplementation of pregnant women and then their infants until 6 months of age significantly reduced the proportion of children sensitized to aeroallergens at age 18 months and the number of primary care visits due to asthma in the treatment group ($p = 0.002$) [214]. A meta-analysis of 3 RCTs showed a reduced risk of offspring wheezing when mothers were supplemented with VitD during pregnancy (relative risk = 0.81, $p = 0.025$) [215]. In adult VitD-deficient asthma patients, VitD treatment appeared to reduce asthma exacerbations only in patients with low 25(OH)D levels but had no significant effect on the general study population [216]. A recent meta-analysis of 7 randomized controlled trials (955 participants) revealed that VitD supplementation significantly reduced the rate of asthma exacerbations requiring treatment with systemic corticosteroids compared to placebo (incidence rate ratio = 0.74, $p = 0.03$). This effect was not detected in the subgroup of patients who had serum 25(OH)D levels of 25 nmol/L or higher at baseline [217].

Inflammatory Bowel Disease (IBD)

IBDs, including Crohn's disease and ulcerative colitis, are chronic inflammatory conditions of the gastrointestinal tract likely arising from a disrupted handling of the antigens present in the gastrointestinal tract by the epithelium and innate immune cells, which leads to a chronic T-cell-mediated infiltration and inflammation of the mucosa. VitD-deficient wild-type mice and VDR- or CYP27B1-knockout mice are more susceptible to experimentally induced colitis and develop more severe symptoms of IBD [52, 56, 218, 219]. Calcitriol treatment of wild-type mice with colitis is associated with a reduced mucosal inflammation accompanied by decreased production of IL-17, TNF-α, and IFN-γ [219]. As discussed earlier, among other tolerogenic effects, VitD suppresses Th1 and Th17 responses and induces Th2 and Treg activity; effects of calcitriol on the innate immune response and intestinal epithelial cells also contribute to its beneficial effects in IBD. VDR-knockout transgenic (VDR-KO/TG) mice that exclusively express VDR in intestinal epithelial cells of the distal ileum and colon display a milder form of colitis and reduced weight loss compared to VDR-knockout mice. Moreover, similar to wild-type mice, upon calcitriol treatment, VDR-knockout mice manifest improved IBD symptoms and increased expression of E-cadherin – an epithelial junctional protein [220, 221]. As VDR is not expressed in immune cells of VDR-KO/TG mice and is exclusive to intestinal epithelial cells, these findings reveal the key role of VitD in these cells and suppression of IBD through maintenance of epithelial integrity. In line with these findings, VDR-knockout mice show impaired epithelial cell tight junctions, which is accompanied by increased incidence and severity of colitis [222]. Another potential mechanism proposed for the protective role of VitD in IBD is helping to maintain the homeostasis of intestinal normal flora via upregulation of antimicrobial peptides (AMPs). For instance, VDR-knockout mice are more susceptible to intestinal *Bacteroides fragilis* infection, which is associated with IBD pathogenesis in humans [223]. As previously explained, calcitriol is crucial for regulation of the NOD2-HBD2 pathway which upregulates the expression of the AMP β-defensin 2. It is shown that attenuated NOD2-HBD2 signaling is associated with increased risk of developing Crohn's disease [224].

Turning to human studies, the prevalence of IBD correlates with increases in latitude in Europe and North America, which is accompanied by lower sunlight exposure and lower VitD status [225]. Patients with active Crohn's disease have lower levels of intestinal VDR expression compared to those in remission [226]. Epidemiologic studies indicate that VitD deficiency is associated with increased risk of IBD, increased disease severity, and increased risk of malignant transformation [227–229]. In a meta-analysis of 14 observational studies, patients with IBD had 64% higher odds of vitamin D deficiency (25(OH)D level of ≤20 ng/mL) when

compared with controls (OR = 1.64; $p < 0.0001$). Latitude did not influence the association between VitD deficiency and IBD ($p = 0.34$) [230]. However, it should be kept in mind that the epidemiologic associations of VitD deficiency with IBD might be the result of defective VitD intestinal absorption in these patients, perhaps before they are told that they have the disease, in which case VitD deficiency would be a consequence – not a cause – of IBD.

Interventional studies in IBD patients have produced encouraging, yet not conclusive results. Supplementation with 1200 IU of cholecalciferol per day insignificantly reduced the risk of relapse in IBD patients from 29% to 13% [231]. Another study reported improved Crohn's Disease Activity Index scores following 24 weeks of VitD supplementation [232]. Two RCTs in IBD patients reported significantly decreased levels of TNF-α, eosinophil sedimentation rate (ESR), and C-reactive protein (CRP) following VitD supplementation [233, 234]. In another RCT, 4000 IU per day of Vit D_3 significantly improved quality of life scores in 10 ulcerative colitis patients [235]. More studies with larger samples are needed to draw conclusions on the potential role of VitD supplementation in management of IBD.

Infectious Disorders

Acute Respiratory Infections

As presented above, calcitriol contributes to innate immune defense via several mechanisms including increased expression of microbicidal proteins by innate immune cells and epithelial cells throughout the body. Airway epithelial cells express both VDR and CYP27B1and, in response to systemic or local calcitriol, secrete AMPs such as cathelicidin and β-defensins, which can kill bacteria- and virus-infected cells.

Hypovitaminosis D has been associated with an increased risk of upper respiratory tract infections (URTIs) in several observational studies [236–238]. A large cohort of 18,883 subjects aged 12 years or older reported that even after adjusting for confounding factors (e.g., smoking history, age, gender, season, asthma diagnosis, etc.), serum 25(OH)D levels were inversely correlated with recent self-reported URTIs. In groups with 25(OH)D levels below 10 ng/ml and above 30 ng/ml, the rate of recent URTIs were 24% and 17%, respectively ($p < 0.001$), with an odds ratio of 1.36. Interestingly, consistent with the aforementioned beneficial role of VitD in asthma control, the correlation between VitD deficiency and URTI was even stronger in subjects with asthma and COPD with odds ratios of 5.67 and 2.26, respectively [239]. Another study in 800 military recruits found that VitD-deficient subjects lost significantly more days from active duty due to URTIs compared to VitD-sufficient subjects [236]. Another cross-sectional survey of 14,108 adults showed a 58% higher risk of URTIs in participants with 25(OH)D levels below 30 ng/ml after adjustment for confounding factors. There was also a linear relationship between VitD levels and cumulative frequency of URTIs, up to VitD levels around 30 ng/ml [240]. VitD deficiency during pregnancy and in newborns is associated with increased risk of URTIs. A study found that 25(OH)D levels below 50 nmol/L increase the odds of developing URTIs in children by 70% [241]. Cord blood 25(OH)D levels below 25 nmol/l were associated with 2.16-fold increased risk of respiratory infections by three months of age in 922 newborns [206].

We previously addressed how VitD can exert a protective effect against viral infections. Recent epidemiological evidence indicates that influenza infection is most common during the first month of winter throughout the world when the VitD levels reach their minimum [242]. Sufficient VitD status is shown to protect against various viral infections including influenza and respiratory syncytial virus (RSV) infections [243]. Infants with cord blood 25(OH)D levels below 20 ng/ml have a significantly higher risk of developing RSV infection during their first year of life compared to those with levels above 30 ng/ml [244].

Nevertheless, VitD supplementation trials have had inconsistent success in preventing the incidence of URTIs and influenza infections in adult populations but perhaps show more promise in pediatric populations [207]. An RCT of monthly 100,000 IU doses of VitD in 322 healthy

adults did not report a reduction in the number or severity of URTI episodes. However, with mean 25(OH)D level of 29 ng/ml, the study population was already VitD-sufficient at baseline [245]. Moreover, in another study that did not detect a beneficial effect for VitD supplementation in URTI prevention, only 29% of the treated subjects reached 25(OH)D levels above 80 nmol/L, indicating inadequate dosing of VitD [246]. In an RCT, daily intake of 4000 IU $VitD_3$ by 140 immunodeficient subjects significantly reduced infectious symptoms and the use of antibiotics over one year in the VitD-treated group [247]. Moreover, 247 VitD-deficient Mongolian children (mean baseline 25(OH)D = 7 ng/ml) treated with VitD-fortified milk demonstrated significantly increased mean 25(OH)D levels (19 ng/ml) and reduced parent-reported URTIs (rate ratio = 0.52) [248]. The RCT of Grant et al. showed that VitD supplementation of pregnant women and then their infants until 6 months of age significantly reduced the number of primary care visits due to URTIs by the age of 18 months [249]. VitD-supplemented subjects self-reported reduced rates of influenza and cold symptoms [250]. A double-blind RCT in schoolchildren using nasopharyngeal swab cultures instead of self-report as the endpoint reported a significant reduction (42%, $p = 0.04$) in the incidence of influenza infections in the VitD-treated arm. The effect was even more pronounced in children who had not been taking VitD supplements before the study [251]. Finally, a recent meta-analysis of 25 RCTs in 10,933 participants aged 0 to 95 years confirmed that VitD supplementation is beneficial in protection against URTIs, particularly in subjects with baseline levels of 25(OH)D below 25 nmol/l (adjusted odds ratio = 0.88 and 0.30, respectively, $p < 0.001$) [252].

Maternal 25(OH)D serum levels during pregnancy are inversely associated with risk of lower respiratory tract infections (LRTIs) in offspring in the first year of life [253]. A cross-sectional study in 16,975 participants showed that after adjusting for demographic factors, season, and clinical data, serum 25(OH)D levels below 30 ng/ml were associated with 56% higher odds of a history of community-acquired pneumonia within the last year compared to levels above 30 ng/ml [237]. A meta-analysis of 12 observational studies concluded that there is an inverse correlation between serum 25(OH)D levels and incidence and severity of LRTIs [254]. VitD supplementation had no effect on preventing the incidence of the first episode of pneumonia in 3046 infants aged 1–11 months. However, severe malnutrition was common in the study population; therefore, the high probability of deficiency of other micronutrients compromises the generalizability of the study results to better-nourished populations [255].

Tuberculosis (TB)

As discussed earlier, the enhancing effect of calcitriol on killing of *Mycobacterium tuberculosis (M. tb)* through induction of innate immune functions has been known for decades. In recent years, several studies have focused on clinical extrapolation of these in vitro findings.

Studies have frequently reported significantly lower VitD status in TB patients compared to healthy subjects. Serum 25(OH)D levels below 30 ng/ml have been associated with increased prevalence of TB [256–258]. A meta-analysis of 25 studies pooling the data of 3599 TB cases and 3063 controls revealed that VitD deficiency is a risk factor for developing TB, not a consequence [259]. Furthermore, it has been shown that VitD-deficient latent TB patients are more likely to proceed to active disease [260].

However, the results obtained from VitD supplementation studies have generally not shown promising responses. A single 100,000 IU oral dose of VitD inhibited the growth of M. tb in whole blood samples of healthy individuals [261]. VitD administration in adjunction with conventional TB regimen significantly reduced the time for sputum acid-fast bacteria smear conversion in TB patients [262], inhibited antigen-stimulated pro-inflammatory cytokine responses [263], and enhanced resolution of lymphopenia and monocytosis [264]. However, two rigorously designed RCTs reported no improvement in sputum conversion time of TB patients receiving high-dose adjunctive VitD therapy, irrespective of whether VitD supplementation managed to significantly increase 25(OH)D serum levels compared to controls or not [265, 266]. A meta-analysis did not

find any positive effect of VitD supplementation in treatment of TB patients [260]. However, it should be noted that most available intervention studies of VitD in TB patients have employed specific paraclinical endpoints such as sputum conversion time rather than clinical endpoints. Therefore, whether VitD administration is clinically beneficial in the treatment of TB should be further addressed in future studies. Martineau et al. found that a single 100,000 IU dose of VitD administered to purified protein derivative (PPD)-positive contacts of active TB patients is able to significantly suppress the growth of M. tb in their whole blood as measured by the BCG-lux assay [261]. The role of VitD treatment in preventing TB infection or activation of latent disease remains to be determined.

VitD Metabolism, Genetic Variations, and Disease

Considering the involvement of several enzymes, receptors, and proteins in the metabolism of VitD, it should be kept in mind that the amount of VitD intake and endogenous synthesis are not the only factors that influence circulating levels of VitD metabolites. Furthermore, circulating level of VitD metabolites is not the sole determinant of the degree of VitD exposure at the cellular level. As was presented earlier, circulating levels of 25(OH)D have been associated with prevalence or severity of various disease conditions. Therefore, every parameter that affects circulating 25(OH)D levels and/or VitD availability and activity at the cellular level for any given serum level of 25(OH)D is expected to correlate with the pathogenesis of various disease states.

As mentioned earlier in this chapter, DBP is the glycoprotein that binds and transports VitD metabolites in the peripheral blood and delivers them to the target tissues. Three allelic forms, DBP-1-1 (Gc1F), DBP-2-1 (Gc1S), and DBP-2-2 (Gc2), have been identified for DBP which show distinguishable geographical and racial patterns of distribution. Different alleles produce DBP phenotypes with remarkably variable DBP serum concentrations and different affinities for binding VitD metabolites, with DBP-1-1 phenotype exhibiting the highest and DBP-2-2 showing the lowest affinity. Studies have reported a link between DBP phenotype with both circulating 25(OH)D concentrations and bioavailability of VitD in target cells [267]. A genome-wide association study of almost 34,000 individuals showed that genetic variations in DBP are independent determinants of serum DBP concentrations, as well as serum 25(OH)D and $1,25(OH)_2D$ concentrations [268, 269]. A possible rationale for the positive correlation between DBP and VitD concentrations could be that DBP facilitates the glomerular reabsorption of 25(OH)D and thereby enhances renal calcitriol synthesis [113]. In line with this evidence, various studies have described the association between DBP polymorphisms and susceptibility to several immune-related disease conditions. As target cells seem to respond more to the free 25(OH)D rather than the DBP-bound form, patients with lower-affinity DBP phenotypes appear to demonstrate enhanced VitD responses. Antibacterial effects of VitD are more pronounced in patients having low-affinity DBP SNP such as Gc1S and Gc2 [270, 271]. A specific SNP in the DBP gene was found to be significantly associated with the prevalence of RA. The haplotype DBP2 was less common in IBD patients [89]. In a large case-control study, the Gc2 genotype was strongly associated with susceptibility to active TB, compared with Gc1 genotype (odds ratio = 2.81, $p = 0.009$) [272]. However, genetic studies have failed to demonstrate an association of DBP polymorphisms and MS and T1DM [89].

More than 70 different SNPs for the VDR gene have been identified, which can influence the abundance and activity of VDR in target cells and affect the immune response. VDR genetic profiling studies have generally reported links between certain polymorphisms and pathologic conditions including TB, IBD, and autoimmune disorders such as T1DM, MS, SLE, and RA [271]. A meta-analysis showed that subjects who are homozygous for the presence of the VDR Fok I polymorphism (the "ff" genotype), which has three more amino acids compared to the "F" form but shows less activity [273], are at higher risk of active TB [274]. Children with ff genotype are at higher risk of developing LRTIs compared to gen-

eral population [275]. Apa I, Bsm I, and Taq I are common SNPs located in the untranslated region of the VDR gene and are thought to affect the expression of VDR via modulation of VDR mRNA stability. A meta-analysis of 13 studies revealed a significant correlation between Apa I polymorphism and susceptibility to IBD [276]. Some studies have also reported that the Bsm I polymorphism (the "B" allele) is associated with higher prevalence of T1DM and TB [277, 278]. An RCT by Martineau et al. demonstrated improved sputum culture conversion rates in 12 VitD-supplemented TB patients with the Taq 1 polymorphism, while such an effect was not observed in the overall study population [266]. In contrast, a meta-analysis in psoriasis patients revealed no significant correlation with VDR polymorphisms [279]. Finally, variations within the genes encoding CYP27B1 have also been associated with autoimmune disorders including T1DM [280].

In summary, exploring the clinical implications of genetic variations in VitD metabolic system is an emerging line of research, and further studies are needed to better define this complex field. Future studies should evaluate the impact of genetic polymorphisms on circulating 25(OH)D concentrations and on clinical disease endpoints at given 25(OH)D levels. This might help explain the large interindividual variations observed in clinical studies in response to the same doses of VitD supplementation in terms of changes in serum 25(OH)D levels. Moreover, better identification of racial and ethnic variability of VDR and DBP genotypes could contribute to establishment of more accurate local guidelines for VitD supplementation and definition of healthy vs. unhealthy VitD status.

Conclusions

In this chapter, we reviewed the diverse roles of VitD in regulation of human immune response. Briefly, the VitD receptor is expressed by virtually all tissues throughout the body, including the immune and inflammatory cells. Monocytes, macrophages, dendritic cells, and activated lymphocytes can locally convert precursor VitD metabolites to the biologically active calcitriol. This forms autocrine and paracrine pathways of VitD metabolism in addition to the endocrine pathway regulated by kidneys. Therefore, the immune system is equipped to both produce and respond to VitD. VitD exerts dichotomous effects on innate and adaptive immune responses. It serves as a potent stimulant of innate defense by upregulating antimicrobial peptides, autophagy, and phagocytosis in macrophages and monocytes upon exposure to pathogens. On the other hand, VitD is thought to be a tolerogenic immunomodulator in adaptive immunity. It is able to suppress maturation and antigen presentation by dendritic cells and shifts them toward a hyporesponsive tolerogenic immature phenotype. It also inhibits proliferation of T cells and downregulates activation of Th1 and Th17 immune responses while promoting Th2 and Treg activity. Finally, calcitriol represses B-cell proliferation and class switching and inhibits the formation of memory and plasma cells and the production of immunoglobulins by B cells.

On this basis, many studies have investigated the potential role of VitD in immune-related pathologic conditions, including autoimmune disorders, chronic inflammatory conditions, and infectious diseases. Many epidemiologic studies have reported strong associations between VitD deficiency and prevalence or severity of various disease states. The results obtained from interventional VitD supplementation trials have been less straightforward, although there still is a paucity of large-scale RCTs that are methodologically equipped to anticipate sources of bias in study design and data analysis. Hopefully, ongoing addition of such rigorously designed RCTs to the available body of evidence will further support and validate the role of VitD as a promising and safe nutrient for prevention and adjunctive treatment of several immune-associated disorders.

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Vitamin A and the Immune System

3

Suyasha Roy and Amit Awasthi

Contents

Key Points

- Vitamin A (VA) and its metabolically active derivative, retinoic acid (RA), play an important role in the development and functions of the immune system; vitamin A deficiency increases the risk of susceptibility to infections.
- RA binds to RAR-RXR, nuclear receptor, expressed on immune cells, and thus regulates the expression of target genes.
- Dendritic cells (DCs) and macrophages are the major producers of RA. RA maintains the intestinal homeostasis and mucosal immunity.
- RA mediates gut migration of T lymphocytes by inducing the expression of gut-homing receptors like $\alpha_4\beta_7$ and CCR9.
- RA reciprocally regulates the differentiation of effector and regulatory T cells, as RA together with TGF-β1 promotes the induction and stability of Tregs while it suppresses the differentiation of Th17 cells.
- RA maintains immune homeostasis, and thus prevents the induction of tissue inflammation in autoimmune diseases.

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Introduction

It was identified in the early twentieth century that dietary constituents play a crucial role in human health and their survival. Among which, vitamin A (VA) is critically important not only for normal growth and development of multicellular organisms but also required for the development and functions of their immune system. VA is one of the most studied nutrients in context of its effect on the immune system. It is a lipid-soluble organic compound that includes retinal, retinol, and β-carotene. It was first proposed by Edward Mellanby and Harry Green that β-carotene and VA are anti-infective agents, which induce protection against infection, as VA-deficient rats showed more susceptibility to infections. This idea was subsequently supported with clinical studies where correcting vitamin A deficiency (VAD) dramatically reduced young childhood mortality and enhanced immune function in endemic regions of malnutrition.

The vitamin A metabolite, retinoic acid (RA), influences multiple populations of both innate and adaptive immunity. VAD leads to loss of vision and dysregulated immune responses, resulting in increased susceptibility to infection. Moreover, VA serves as an adjuvant, which has been shown to enhance vaccine efficacy by eliciting protective immune responses for vaccines. However, the overdose of VA can lead to liver toxicity [1]. Of particular interest to the present chapter is that VA and its metabolites can influence multiple immune cell lineages and modulate their effector functions, such as immune tolerance, lymphocyte trafficking, gut homing, and tissue inflammation. In this chapter, we would highlight the recent advances of VA and its metabolite in the development and functions of immune cells, and the mechanism through which retinoic acid regulates the outcome of immune responses in infection and autoimmunity.

Metabolism and Biology of Vitamin A

VA is a lipophilic micronutrient acquired from diet by ingestion of all-trans-retinol, β-carotene, and retinyl esters from plant and animal food. Humans are not able to synthesize VA and therefore heavily depend upon VA supplementation. Since retinyl esters and retinol are not biologically active forms, they are enzymatically converted to RA, a biologically active metabolite of VA. Retinyl esters are packaged into chylomicrons by intestinal epithelial cells and exported in the lymphatic system and portal vein. Retinyl esters are taken up and stored in the stellate cells of the liver (Fig. 3.1) [2, 3]. The hydrolysis of retinyl esters and esterification of retinols are carried out by the enzymes retinyl ester hydrolase (REH) and lecithin retinol acyltransferase (LRAT), respectively [4]. The stored retinyl esters or retinol is released in the circulation bound by either retinol-binding protein (RBP) during homeostasis or by serum amyloid A during infection [5, 6]. The circulating retinol bound by RBP in the cytosol is taken up by membrane transporter stimulated by retinoic acid 6 receptor (STRA6); retinol crosses the membrane by passive diffusion while leaving behind RBP at the extracellular side of the membrane [6, 7].

After its uptake by intestinal epithelial cells, alcohol dehydrogenases (ADH) convert retinol to retinaldehyde (retinal) by an oxidation step (Fig. 3.1). Once converted into retinal, it is further transformed into *all-trans*-retinoic acid (*at*RA) by one of the three isoforms of retinaldehyde dehydrogenases RALDH1, RALDH2, and RALDH3, which are encoded by genes *aldh1a1, aldh1a2,* and *aldh1a3*, respectively (Fig. 3.1) [8]. *at*RA is physiologically most abundant and well studied as compared to other isoforms, 9-*cis*-RA and 13-*cis*-RA, and is responsible for most of the biological effects of VA. Retinaldehyde dehydrogenases are crucial for *at*RA synthesis, as *Aldh1a2* and *Aldh1a3* deficiencies in mice lead to impaired fetal and embryonic development. The function of RALDH1 is redundant; the viability of *Aldh1a1*-deficient mice suggests its non-redundant role in RA metabolism [9]. RALDH is selectively expressed and tightly regulated in non-immune and immune cells in the gut where it contributes to the production of RA in the intestinal microenvironment.

Among other isoforms of RA such as *all-trans*-RA, 9-*cis*-RA, 13-*cis*RA, 11-*cis*RA, and 9,13-*dicis*RA, *at*RA is the most abundant bio-

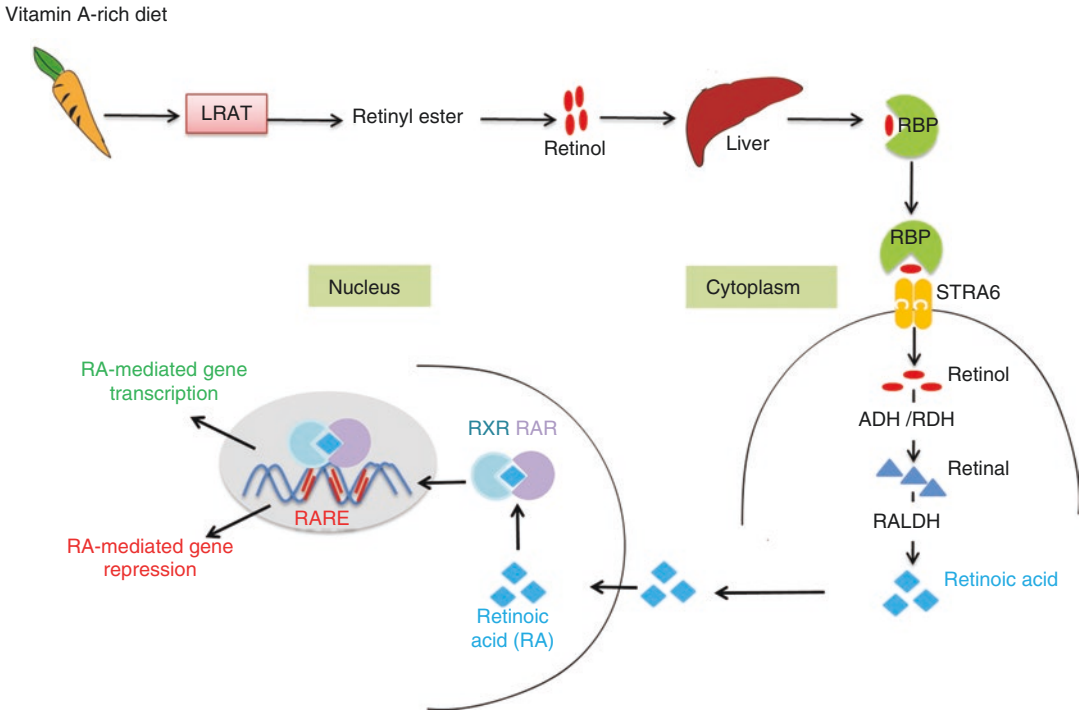

Fig. 3.1 Metabolism of vitamin A and retinoic acid (RA) signalling. Dietary vitamin A gets absorbed by the small intestine and transported to the liver for storage via blood circulation. Through enzymatic actions, vitamin A converts into retinyl ester, which further gets converted into retinol. Retinol-retinol binding protein (RBP) complex through blood circulation enters into the cell. Upon retinol entry into the cell, it is reversibly oxidized to retinal by alcohol dehydrogenase (ADH), which then irreversibly metabolizes to retinoic acid (RA) by retinal dehydrogenases (RALDH). RA exerts its effect through RAR/RXR heterodimer, which induces conformational changes to promote histone acetylation and activation of transcription. In the absence of RA, RAR/RXR binds to the DNA along with the co-repressors to induce transcriptional repression through histone deacetylation

logically active isomer form [10]. Mechanistically, RA translocates into the nucleus where it binds to nuclear receptors, including retinoic acid receptors (RARs) α, β, and γ; retinoid X receptors (RXRs) α, β, and γ; and the peroxisome proliferator-activated receptors (PPARs) β/δ. Both *all-trans*-RA and 9-*cis*-RA bind to RAR, but only 9-*cis*-RA binds to RXR although 9-*cis*-RA has been detected only in the pancreas [11]. Upon binding with RA, RAR forms a heterodimer with RXR, which then binds to retinoic acid responsive elements (RARE) in the genome to further regulate gene expression of target genes. RXR activated by the binding of 9-*cis*-RA also forms dimer with other nuclear receptors such as PPARs, liver X receptor (LXR), etc. [12]. Moreover, RA bound to RAR acts as an epigenetic regulator by mediating histone modifications and DNA methylation through chromatin modifications mediated by the recruitment of co-activators thus influencing translation as reported by studies in embryonic stem cells. In the absence of RA, RAR/RXR heterodimer recruits co-repressors such as NCoR or SMRT, which in turn recruit histone deacetylases inhibiting the transcription of target genes. Binding of RA to RAR/RXR heterodimer displaces co-repressors allowing the recruitment of co-activators such as p300 and CBP, resulting in the activation of transcription of target genes [13].

The functions of RA are tightly regulated. RA signalling can be terminated by the degradation of RA into oxidized metabolites such as 4-oxo-RA through cytochrome P450 family 26 enzymes (CYP26A1, CYP26B1, and CYP26C1), which are found in several tissue and cell types. RA forms a negative loop where it directly induces CYP26A1, thereby initiating its own degradation.

CYP26 enzymes regulate the local concentrations of RA by regulating the tissue-specific clearance of RA [14]. Differential expression and activity of CYP26 enzymes in specific tissues alters *at*RA signalling and its effector functions. Animals deficient in cyp26a1 and cyp26b1 enzymes display severe multiple developmental abnormalities [15]. RA binds to three different lipid-binding proteins such as cellular retinoic acid-binding protein I and II (CRABPI and CRABPII) and fatty acid-binding protein 5 (FABP5) in the cytosol [16]. Binding of RA to its lipid-binding protein partners is crucial for its nuclear translocation. RA has differential binding affinities, as CRABPII has a higher affinity for RA as compared to FABP5. Moreover, CRABPII bound RA complex is targeted to the nuclear RAR receptor, while FABP5 bound RA complex is targeted to PPARβ/δ receptor, suggesting that RA-induced effector functions are differentially regulated and dependent on its lipid-binding protein. Both RAR and PPARβ/δ play important physiological roles in various tissues depending upon their relative expression levels; nonetheless, RAR signalling pathway is predominantly induced by RA [17, 18]. Taken together it can be suggested that *at*RA signalling is tightly regulated at multiple levels by the differential expression of its nuclear receptors, lipid-binding proteins, RA synthesizing, and degrading enzymes.

The transportation of RA between cells is not clearly identified. However, studies have suggested that RA can be transported through membrane contact via specific transporter channels or by the transfer of cell membrane components. RA can be packaged into lipoprotein particles or exosomes. Additionally, RA can be directly bound to serum proteins such as apolipoprotein A1 and albumin, which are transported between cells [19, 20].

Vitamin A and Innate Immune Cells

Dendritic cells (DCs) and macrophages are the primary cell types of the innate immune system and are the major producers of RA. The ability to convert VA into RA is dependent on the expression of aldehyde dehydrogenase 1a (ALDH1a), which is expressed in cells of lamina propria (LP) and mesenteric lymph nodes (MLN). In addition, DCs and macrophages located in MLN, Peyer's patches (PP), LP, and gut-associated lymphoid tissue (GALT) are the major producers of RA, as they constitutively express RALDH2. Among the gut DCs, ALDH activity is restricted to $CD103^+$ DCs but not on $CD103^-$ DCs or any other cell types in the gut. In addition to the gut, DCs resident in the lung and skin also express *aldh1a2* maintaining immune responses at barrier sites. DCs located at the proximal end show higher ALDH activity as compared to DCs located in the distal end of the gut. Thus, $CD103^+$ DCs in the proximal part of the intestine are better producers of RA as compared to those in the distal end of the small intestinal or colonic DCs [21].

RA signalling regulates the induction of ALDH1a in DCs and macrophages. RA produced by DCs and macrophages itself acts in a feed-forward loop to induce ALDH1a expression. VAD in animals reduces expression of ALDH1a. In addition to RA, short-chain fatty acids such as butyrate also suggested to induce ALDH1a expression in intestinal DCs [22]. TLR ligands particularly TLR1/2 and TLR5 trigger ALDH1a expression in DCs, which produce *at*RA [23]. TLR2-induced ALDH1a expression enhances the expression of suppressor of cytokine signalling 3 (SOCS3), which suppresses IL-23 production in DCs [24], suggesting that ALDH1 suppresses pro-inflammatory cytokine production by DCs. In addition to TLRs, cytokines such as GM-CSF, IL-4, and IL-13 increase the expression of ALDH1a in DCs and macrophages [25]. Cellular signalling pathways required for the induction of ALDH1 in innate immune cells such as macrophages and DCs include the mitogen-activated protein kinase (MAPK), p38MAPK [26]. In addition to MAPK, other signalling pathways such as β-catenin and Wnt signalling pathways are also crucial for the induction of ALDH1a expression in intestinal DCs [27]. Thus the interplay between cytokines, signalling pathways, inflammatory conditions,

and RA regulates the expression of ALDH1a in DCs and macrophages.

RA profoundly affects the function of macrophages by regulating the production of cytokines such as TNF-α, IL-1, IL-6, and IL-12 as well as nitric oxide [28, 29]. Furthermore, treatment of T-cell-macrophage co-culture with RA reduced IFN-γ production while increasing the secretion of IL-4 from T cells, suggesting that RA modulates Th1/Th2 differentiation pathways. Moreover, RA suppressed the expression of IL-12, which is known to induce IFN-γ from T cells [28].

In addition to macrophages and DCs, RA also plays a critical role in the differentiation of neutrophils. RA binds to its receptor enhancing the expression of the genes which promote the development and maturation of neutrophils in the bone marrow [30]. RA also positively cooperates with enhanced superoxide production by neutrophils [31], as VAD results in impairing the development and frequency of neutrophils. The defect in the frequency of neutrophils in RA-deficient animals could be restored by the supplementation of RA [32]. Altogether, these observations clearly implied that RA is an important modulator for the functions of innate immune cells.

Vitamin A and Dendritic Cell Functions

DCs are specialized sentinels of the immune system. They process and present antigens to naïve T cells to initiate adaptive immune responses. DCs have the ability to migrate to draining lymph nodes and produce cytokines, which help in the differentiation of T cells into various effector subsets. VA metabolite, RA, regulates the differentiation and function of DCs including antigen processing and presenting capacity in multiple ways. It has been demonstrated that RA promotes the development and differentiation of splenic $CD8^-$ $CD11b^+$ $CD4^-$ $Esam^{high}$ DCs and intestinal $CD11b^+$ $CD103^+$ DCs without affecting other subsets of DCs [33]. $CD8^-$ DCs include both $CD4^+$ DC and DN DC, which express higher levels of CD11b, DCIR2, Sirpα, and endothelial cell-selective adhesion molecule (ESAM). Both of these subsets of DCs were shown to present antigens to $CD4^+$ T cells *in vitro* and *in vivo*. The effect of RA on DC functions was investigated in VAD mice and in mice treated with pan-RAR antagonist. In both settings, $CD8^-$ $CD11b^+$ $CD4^-$ $Esam^{high}$ and $CD11b^+$ $CD103^+$ DCs were found to be decreased while $CD8^+$ DCs were increased. These findings suggest that RA modulates the frequencies of different subsets of DCs to control the differentiation and functions of $CD4^+$ T cells [34]. In DC developmental pathways, Notch signalling has been shown to be essential for the generation of both splenic $CD8^-$ $CD11b^+$ $CD4^-$ $Esam^{high}$ and intestinal $CD11b^+$ $CD103^+$ DC populations [35]. RA signalling influences the binding of Notch ligands such as delta-like ligands 1, 3, and 4, as well as jagged 1 and 2 to Notch receptors on DCs. Upon binding, Notch receptor is activated followed by proteolytic cleavage of the intracellular Notch domain by metalloproteinases and γ-secretases [36]. In addition, it has been shown that RA promotes the development of $CD8^-$ DCs by stimulating the expression of LTαβ, LTβR, EBI2, or its ligands [35].

RA produces anti-inflammatory effects on intestinal DCs by targeting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). RA has the potential to cause both anti- and pro-inflammatory effects. The combination of RA and IL-4 enhances the secretion of SOCS3, which would suppress the production of pro-inflammatory cytokines, e.g., IL-6, TNF-α, and IL-12p70 [37]. RA, however, together with IL-15, activates the JNK pathway to enhance the induction of pro-inflammatory cytokines IL-12p70 and IL-23 by DCs [38]. In addition to its effect on cytokine production in DCs, RA in combination with pro-inflammatory cytokines such as TNF-α and IL-1β would enhance antigen-presenting functions of the skin's DCs by upregulating the expression of major histocompatibility complex (MHC) class II molecules and CD86 [39]. In contrast, RA has been shown to decrease the expression of MHC-II and costimulatory molecules, CD80 and CD86, on splenic DCs in experimental autoimmune encephalomyelitis (a mouse model of human

disease multiple sclerosis) [40]. These observations suggest that RA has differential effects on different types of DCs and the contrasting role of RA in mediating pro- or anti-inflammatory phenotypes on DC relies on local microenvironment cues and cytokine milieu.

RA modulates mucosal DC functions. Of note, it induces the secretion of transforming growth factor beta (TGF-β) 1 and gut-homing receptors, CD103, on T cells, while depletion of RA reduces the surface expression of CD103 on mucosal DCs [41]. In addition, RA promotes migratory functions of DCs by inducing the expression of matrix metalloproteinases (MMP) and suppressing the expression of MMP inhibitors known as tissue inhibitor of matrix metalloproteinase (TIMP-1, TIMP-2, TIMP-3) [42]. RA also inhibits the expression of the adhesion molecule CD11a on DCs. RA promotes the differentiation of migratory DC precursors into plasmacytoid DC (pDC) and conventional DCs that preferentially develop into $CD103^+$ DCs in intestinal LP and spleen by inducing the expression of mucosal homing receptor $\alpha_4\beta_7$ on bone marrow-derived $B220^+$ $CD11c^{int}$ DC. In inflammatory and infectious conditions, RA also induces the chemokine receptor CCR9 on DCs [35]. VA also influences the follicular dendritic cells (FDCs) in the germinal centre which facilitate the B-cell polarization for IgA production. RA binds to receptors on FDCs and upregulates the expression of chemokines CXCL13 and BAFF which enhance the secretion of TGF-β1 [43].

However, cAMP suppresses RA synthesis by inhibiting the expression of *aldh1a2* in DCs. cAMP is induced by prostaglandin E_2 (PGE_2) serving as a central negative regulator of RALDH expression in DCs [44]. Also, the *aldh1a2* expression and RA synthesis are decreased in intestinal and MLN DCs in mouse model of colitis induced by T-cell transfer in mice and during *Trichuris muris* infection due to the inflammatory milieu [45, 46]. PGE_2 inhibitor can be used for inducing gut immune responses by intestinal DCs.

VA is crucial for the maintenance of intestinal homeostasis and mucosal immunity. It is important for the proliferation and maturation of epithelial cells in the gut as well. VAD induces keratinization and increases the synthesis of large fucosylated glycopeptides which increase the susceptibility to intestinal pathogens leading to dysbiosis. VAD leads to a reduction in the antimicrobial peptide defensins and RegIIIs in the inner mucus layer, whereby the microbes are allowed to come in closer contact with the immune cells at the epithelial surface. Moreover, VAD alters the expression of cell adhesion molecules and microbial receptors which affect the intracellular transportation and therefore processing and presentation of antigens and the degree of microbial uptake. Moreover, VAD increases the interaction between microbes or allergens and immune cells such as DCs, macrophages, and stromal cells in the LP by impairing permeability at the apical junction [43].

Vitamin A and T-Cell Activation

The balance between effector and regulatory T cells is crucial to maintain an immune homeostasis. RA has been implicated in regulating T-cell activation and differentiation. In this section, we describe the effect of RA on the interplay between effector and regulatory T cells during tissue inflammation.

RA has been shown to specifically prevent activation-induced apoptosis of T cells and antigen-specific deletion of immature $CD4^+CD8^+$ double-positive thymocytes from T-cell receptor transgenic mice. Moreover, this effect was more potently induced by 9-cis-RA as compared to *at*RA, implying that RXRs participate in regulating T-cell development [47]. It is suggested that retinoids are important cofactors, which together with TCR activation promote T-cell activation, as the presence of retinoids helps in the transition from G0 to G1 phase of cell cycle in T cells [48]. It is also shown that RA is essential for TCR-mediated activation of $CD4^+$ T cells through RARα signalling, as RARα-deficient T cells had reduced T-cell activation and proliferation as compared to wild-type T cells. This is supported mechanistically with the fact that RA stabilized and enhanced the expression of transcription factor NFAT, which is crucial for activation-induced

IL-2 production in T cells [49]. NFAT-IL-2 axis is well established in TCR-mediated activation and proliferation of T cells, as both NFAT- and IL-2-deficient T cells failed to induce T-cell activation and proliferation [49]. In support of these observations, VAD mice were shown to have reduced levels of NFAT proteins, which restore to normal levels upon administration of RA [49], indicating that RA is an important inducer of T-cell activation and proliferation.

As T cells get activated by TCR-CD3 signalosome, it triggers intracellular Ca^{2+} mobilization, resulting in the nuclear translocation of NFAT leading to sustained T-cell activation. These T-cell activation events were found to be defective in RARα-deficient mice and mice treated with pan-RAR antagonist [50]. It has been demonstrated that VA is absolutely required for anti-toxoplasma Th1 immunity, as depletion of VA in natural model of VA insufficiency in which mice received no VA in their diet starting day 14.5 in utero not only failed to express T-bet-IFN-γ axis but also failed to induce expression of $\alpha_4\beta_7$ on $CD4^+$ T cells [50]. These observations indicated that the dietary VA is absolutely crucial for the induction of protective T-cell immunity in *Toxoplasma gondii* infection. This is consistent with the previous findings suggesting that RA acts as an adjuvant during infection [51]. The defective Th1 immunity in mouse model of *Toxoplasma gondii* infection was further suggested to be due to a defect in T-cell activation pathway, confirming that the VA is essential in T-cell activation. More precisely, RARα-deficient T cells, as compared to wild-type counterpart, failed to induce activation. This defect of T-cell activation was persisted and could not be repaired even with the supplementation of exogenous IL-2, which is a known T-cell proliferative cytokine [49]. RARα-deficient T cells also failed to induce the expression of T-cell activation markers such as CD69, CD25, and CD71. Although the proximal TCR signalling components were equally activated in wild-type as well as RARα-deficient T cells, AKT and protein S6 (pS6) phosphorylation were found to be severely defective in RARα-deficient T cells. Consistently, the activation of mTOR kinase pathways was impaired in RARα-deficient T cells [52]. This clearly implied that VA particularly contributes to late signalling, but not early, events in T-cell activation pathways. In addition to AKT and pS6, RARα-deficient T cells also show decreased activation of phosphoinositide-specific phospholipase C (PLCγ) and extracellular signal-regulated kinase-1 (ERK) upon activation [53].

IL-2 is produced by activated T cells to further support T-cell proliferation and therefore T-cell activation. It has been shown that RA can contribute to TCR-mediated T-cell proliferation in an IL-2-dependent manner [54]. T-cell activation is required for the activation of NFAT family transcription factors, which, in turn, support the production of IL-2 and contribute to the activation of effector $CD4^+$ T cells. VAD leads to reduced expression of NFAT in T cells. Moreover, it has been demonstrated that one of the isoforms of NFAT, NFATc2, makes a complex with RARα-RXR to induce the expression of CCR9, while the other isoform of NFAT, NFATc1, impairs the expression of CCR9. Altogether there is a molecular interplay between RA and NFAT in T-cell activation and associated effector functions. Ca^{2+} mobilization is another essential component of T-cell activation. TCR-CD3 engagement induces Ca^{2+} mobilization, which induces NFAT translocation into the nucleus. The Ca^{2+} mobilization was impaired in RARα-deficient T cells, which also show a defective NFAT activation [49]. In addition to NFAT, TCR-mediated signalling in T-cell activation also activates the AP-1 family transcription factor, c-Jun, which binds to DNA to induce the response to stress. RA-RARα signalling regulates the DNA binding of c-Jun during T-cell activation. Non-nuclear activities have also been described for RARα. RARα was found to be associated with certain scaffold proteins, which are located in the membrane or cytoplasm facilitating T-cell activation. Another non-nuclear function of RARα is to regulate the expression of phosphatase MKP1, an essential molecule in T-cell activation [55]. Although it is clear that RA plays a role in T-cell activation, it is not well-understood how RA sequentially induces signalling events that regulate TCR-mediated activation of T cells.

Vitamin A, Cellular Migration, and Gut Homing of T Cells

To mount a specific immune response, the immune cells particularly T cells migrate to the site of injury or infection. T-cell migration is governed by chemokines and chemokine receptor signalling, which help T cells to migrate and home. Above, some homing receptors of T cells, which allow T cells to migrate and home to specific sites during inflammation, were described. Gut-homing phenotypes on T cells are induced by DCs, which synthesize RA. The importance of VA for inducing a gut-homing property on T cells is clearly evident in VAD mice in which the trafficking of both $CD4^+$ and $CD8^+$ T cells to the gut is severely impaired. Among other DCs, DCs from gastrointestinal tract and gut-associated lymphoid tissues (GALT) were shown to induce gut-homing markers such as $\alpha_4\beta_7$ and CCR9 on T cells in a RA-dependent manner, as the neutralization of RA by its antagonist in GALT DCs-T-cell co-culture failed to induce $\alpha_4\beta_7$ and CCR9 on T cells (Fig. 3.2). These observations suggest that GALT DCs generate RA, which is essential to induce the expression of gut-homing receptors on T cells. CCR9 binds to its ligand CCL25, expressed by intestinal epithelial cells, whereas $\alpha_4\beta_7$ integrin binds to its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1), expressed by high endothelial venules in GALT [56].

$CD103^+$ DCs found in PP and MLNs are the major producers of RA. They express higher levels of RALDH1 and RALDH2 and thus play a critical role in inducing gut-homing receptors, $\alpha_4\beta_7$ and CCR9, on T cells in mucosal tissues (Fig. 3.2). Together with $CD103^+$ DCs, $CX3CR^+$ macrophages and stromal cells residing in GALT also serve as important sources of RA for T cells and contribute to inducing gut-homing receptors $\alpha_4\beta_7$ and CCR9 on both $CD4^+$ and $CD8^+$ T cells in the absence of cytokines and inflammation. Recent research showed that RA-RARα complex binds to RAR-response elements (RARE) within the regulatory region of the gene that encodes α_4[56]. Similarly, a binding site of RARE was

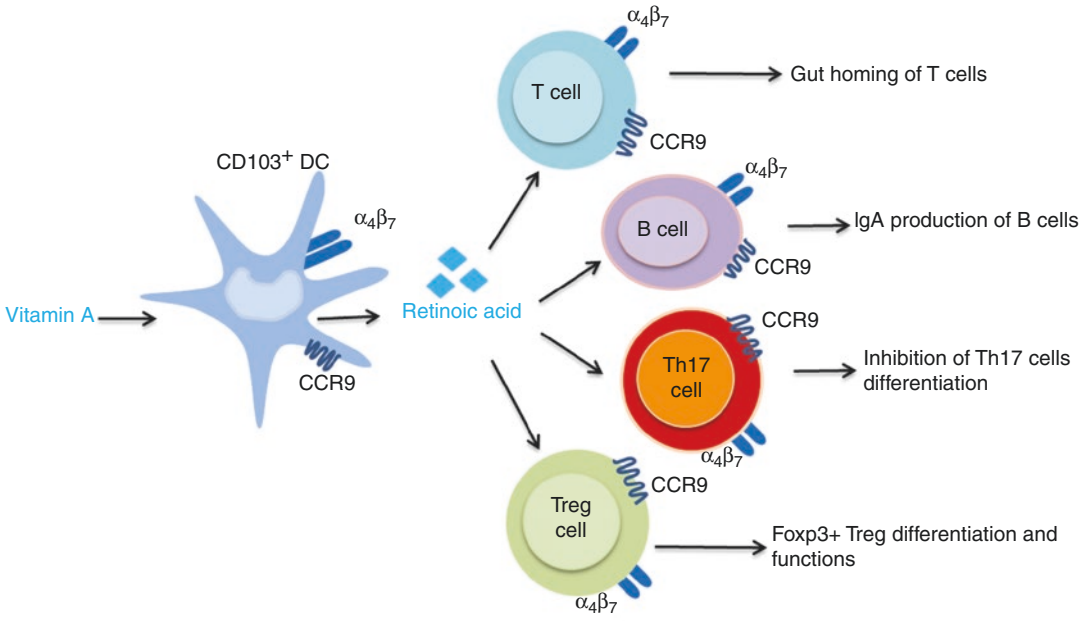

Fig. 3.2 Effect of RA on lymphocytes and their gut homing. Intestinal $CD103^+$ DCs metabolize vitamin A into metabolically active RA and modulate T-cell development and gut homing. RA exposure upregulates surface expression of $\alpha_4\beta_7$ integrin and CCR9 receptor on lymphocytes to facilitate their guthoming. RA potentiates B-cell production of IgA. During T-cell differentiation, the presence of RA suppresses the differentiation and functions of Th17 cells while promoting $FoxP3^+$ regulatory T cells (Tregs) differentiation

also found in the promoter region of CCR9 gene locus [56]. More precisely, RARα forms a heterodimer complex with RXR, which then binds to RARE on the CCR9 promoter to induce its surface expression. Under inflammatory condition, GALT DCs produce RA, which induces the formation of heterodimer of α_4 integrin with β_7, followed by the binding of $\alpha_4\beta_7$ to vascular cell adhesion molecule-1 (VCAM-1) endothelium [56]. RA + DCs induce the gut-homing receptors on effector T cells and also on Foxp3$^+$ Tregs. The induction of $\alpha_4\beta_7$ and CCR9 on Foxp3$^+$ Tregs is dependent on the activation of transcription factor BATF [57]. It has been demonstrated that RA preferentially induces gut homing of lymphocytes, by inhibiting the expression of skin-homing receptors such as E-selectin on T cells and therefore facilitating their migration to the gut [58].

Vitamin A and Regulatory T-Cell Functions

Foxp3$^+$ T cells are specialized subset of Tregs maintaining peripheral tolerance and immune homeostasis. Natural occurring Foxp3$^+$ Tregs (nTreg cells) develop during maturation of T cells in thymic selection processes within the thymus. In addition, the generation of Foxp3$^+$ Tregs can also be induced extrathymically in the periphery and hence referred to as induced Tregs (iTregs). Both natural occurring and induced Tregs profoundly regulate the effector T-cell functions [59]. iTregs can be generated from naïve T cells by chronic exposure of antigen or exposure to metabolites at mucosal sites [59]. TGF-β1 and IL-2 are essential cytokines that induce the development of iTregs from naïve T cells, as both TGF-β1 and IL-2 deficiencies lead to autoimmune inflammation due to lack of Tregs. In addition, it was shown that RA acts as a cofactor that would support the generation of iTregs along with TGF-β1/IL-2. RA not only enhances the generation of TGF-β1-induced iTregs but also maintains the stability of nTregs under inflammatory conditions [59]. Multiple mechanisms have been proposed by which RA can promote iTreg induction. One of the mechanisms suggests that RA induces Foxp3 in peripheral non-Foxp3 naïve T cells by inhibiting the production of cytokines such as IL-4, IL-21, and IFN-γ from memory T cells, which are known to suppress TGF-β1-induced Foxp3 expression [60]. The activation of GALT memory T cells (CD4$^+$CD44^{high}) in the absence of RA leads to the expression of CYP26B1 (RA-degrading enzyme), which degrade RA and decrease the expression of CCR9 on T cells. TGF-β1 promotes RA signalling by inhibiting the expression of CYP26B1 [61]. Moreover, TGF-β1 has been shown to synergistically enhance the effect of RA on the generation of iTregs. Similar to TGF-β1, RA may increase TGF-β1-induced generation of iTregs by enhancing TGF-β1 signalling via activation of Smad3 [62]. RA mediates binding of RAR/RXR heterodimer to RA response element (RARE) in the enhancer 1 (CNS1) region of the Foxp3 gene, allowing the binding of activated Smad3 to CNS1 of Foxp3. TGF-β1-induced activation of Smad2/Smad3 is a prerequisite for RA-mediated expansion of iTreg cells, as blockade of TGF-β1 signalling diminished the effect of RA on iTreg generation. TGF-β1 triggers Smad3 activation allowing the binding of RA, which then increases the total expression of Smad3 in iTreg generation [62]. Taken together these observations indicate that RA alone is insufficient to induce Foxp3 expression in the absence of TGF-β1 in iTregs. Along with TGF-β1, RA can enhance the stability of iTregs by sustaining the expression of Foxp3 and therefore maintaining the suppressive function of Tregs. The role of RA in the generation of Tregs has been demonstrated in the mouse model of *Listeria monocytogenes* infection, as administration of pan-RAR antagonist LE540 significantly reduced the number of mucosal Foxp3$^+$ Tregs. Similarly, VAD mice were defective in the generation of iTregs [63].

DCs activate naïve T cells into various effector and regulatory T-cell subsets. Different DC subsets have been identified, which preferentially induce the generation of iTregs. Evidence of how DCs modulate the generation of iTregs came from *in vitro* findings that DCs from MLN and LP relatively induce higher differentiation of iTregs as compared to splenic DCs in the presence of TGF-β1 [64]. Further fractionation of DC population based on the surface expression of CD103 revealed

that CD103$^+$, but not CD103$^-$, DCs from MLN and LP induce the differentiation of iTregs [64]. It was further demonstrated that CD103$^+$ GALT DCs produce RA, which cooperatively with TGF-β1 enhances the differentiation of iTregs. It was shown that RA promotes iTreg generation by inducing the expression of Arginase I in DCs. Inhibition of TGF-β1 or RA in the co-culture of naïve T cells with CD103$^+$ GALT DCs abrogated the generation of iTregs, suggesting that both TGF-β1 and RA are produced by CD103$^+$ GALT DCs to support the generation of iTregs [64].

As discussed above, RA signalling is crucial for inducing gut tropism on Tregs, which play a role in tolerance against food antigens. Consistently, VAD results in developing food allergies, inflammatory bowel disease (IBD), and celiac disease. Additionally, RA appears to maintain barrier function of gut epithelium. Administration of RA has shown beneficial effects in a dextran sodium sulphate (DSS)-induced colitis model [65]. Generally, oral antigens are taken up by CX3CR1$^+$ macrophages as well as CD103$^+$ DCs, which then migrate towards MLNs in a CCR7-dependent manner. Upon reaching MLN, DCs subsequently present antigens to naïve T cells to differentiate into either pro-inflammatory Th17 or immunosuppressive Foxp3$^+$ Tregs. The generation of Th17 cells and Tregs within the gut relies upon the microenvironment and cytokine milieu at the site. Presence of RA within the microenvironment suppresses the generation of Th17 cells and its associated tissue inflammation by shifting the balance towards the generation of Foxp3$^+$ iTregs. RA would mediate the expression of $\alpha_4\beta_7$ and CCR9 on Foxp3$^+$ Tregs. These $\alpha_4\beta_7^+$ CCR9$^+$ Foxp3$^+$ Tregs migrate to LP of the small intestine where they produce IL-10 to maintain immune homeostasis. Thus, RA is critical for the induction of gut tropism in both Foxp3$^+$ Tregs and Foxp3$^-$ IL-10-producing Treg cells which maintain tolerance towards food antigens. CCR9-deficient Foxp3$^+$ Tregs fail to induce tolerance because they lack gut-homing receptors [21].

An inflammatory environment makes iTregs and nTregs unstable. The pro-inflammatory cytokine IL-6 not only suppresses the differentiation of naïve T cells into iTregs but also inhibits suppressive functions of nTregs by making effector T cells refractory to Treg-mediated suppression. Zhou et al. have addressed the issue of Tregs stability and demonstrated that *at*RA maintains the stability and suppressive functions of Tregs via downregulation of IL-6 receptor (IL-6R) expression. Tregs treated with RA suppressed the established collagen-induced arthritis. This further explains that RA suppresses IL-6R expression induced by TGF-β1, and thus inhibits the differentiation of Th17 cells [62]. These observations were further validated in human settings where treatment with RA induced stability of human nTregs even in the presence of inflammatory environment via downregulation of IL-6 and IL-1 receptors [66]. Mechanistically, it is demonstrated that *at*RA treatment affects the epigenetic modifications in Foxp3 locus to provide durable stability and suppressive functions of nTregs. RA induces the expression of microRNA miR-10a, which enhances the expressions and functions of Bcl-6 and Ncor-2 and impairs the stability of iTregs [67]. DCs and macrophages from sites other than GALT also produce RA and influence T-cell functions. CD103$^+$CD11b$^+$CD24$^+$ DCs in the skin and CD11c$^+$ F4/80$^+$ macrophages in the lungs produce RA, which has been shown to potentiate the effect of TGF-β1 in the differentiation of Foxp3$^+$ iTregs. IL-10, an anti-inflammatory cytokine, suppresses T cells and antigen-presenting functions in order to control tissue inflammation. It has been demonstrated that RA enhances the production of IL-10 from Tregs to induce their suppressive functions during inflammation. Also, RA would induce the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) on the surface of Foxp3$^+$ Treg cells, which indicates suppressive functions of Tregs. CTLA4-deficient Tregs failed to control tissue inflammation.

Effect of Retinoic Acid on Differentiation of Effector T Cells and Their Functions

Upon antigenic stimulation, naïve T helper (Th) cells differentiate into different effector Th cell subsets. Cytokines play a role in the differentia-

tion of Th cells. Distinct Th cell subsets such as Th1, Th2, Th9, and Th17 have been described and defined based on the expression of their lineage-specific transcription factors, effector functions, and cytokine secretion. RA plays an important role in maintaining immunological tolerance by influencing the differentiation of effector Th subsets (Fig. 3.3).

In both mice and humans, RA is critical for the differentiation and functions of Th1 cells. Initial observations suggested that RA exerts its effects directly on T cells to modulate the differentiation of Th1 cells. Lower doses of RA were found to suppress Th1 differentiation by shifting it towards Th2 differentiation pathway [68]. Similarly, *at*RA treatment ameliorated sulphonic acid-induced colitis by shifting Th1 to Th2 cells [68]. Consistently, RA has been shown to induce Th2 differentiation. It was later found that RA supports the differentiation and functions of Th1 cells with the observation that VAD led to increased burden of *Toxoplasma gondii* infection associated with diminished frequency of Th1 cells [50]. Moreover, in a mouse model of celiac disease, RA together with IL-15 induced disease pathogenesis associated with increased numbers of Th1 cells (Fig. 3.3). RA and IL-15 modulate DCs, thereby increasing the production of IL-12 and IL-23 in a JNK-dependent manner to support Th1 differentiation [38]. Both these cytokines have been shown to interfere with Treg stability and functions. RA would contribute to Th1/Th17

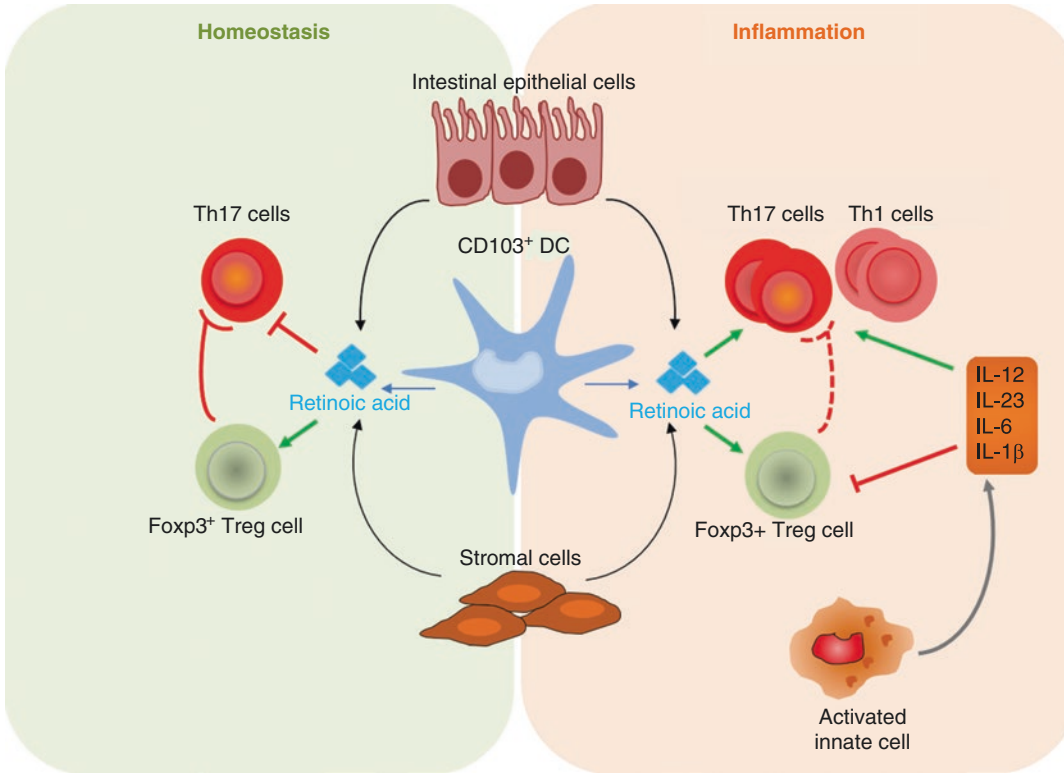

Fig. 3.3 Role of retinoic acid in immune homeostasis and tissue inflammation. Retinoic acid synthesizing cells such as CD103+ DCs, intestinal epithelial cells, and stromal cells convert dietary vitamin A into metabolically active RA. At steady state, RA mediates immune homeostasis by promoting the generation and functions of iTregs cells and suppressing the generation of pathogenic Th17 cells in the intestine and gut-associated tissues (left side). Upon infection, RA helps in promoting the generation of Th17 cells, which are crucial in maintaining gut barrier functions. During infection or inflammation, inflammatory cytokines produced by activated innate cells such as macrophages and DCs create inflammatory milieu, which together with RA potentiate the development and functions of Th17 and Th1 while suppressing the development and functions of iTregs

generation while suppressing the induction of Tregs by increasing the production of IL-12 and IL-23 [38]. More precisely, the cell-intrinsic role for RA in Th1 differentiation and functions has been demonstrated in a mouse model where dominant negative (dn) form of RARα was expressed in T cells to define the role of RA-RARα axis in Th differentiation [69]. Brown et al. clearly demonstrated that ligand-dependent functions of RA are crucial for Th1 cell differentiation and functions. dnRARα T cells are defective in the expression of T-bet, a master transcription factor of Th1 cells, leading to diminished production of IFN-γ. Moreover, an impaired RA-RARα signalling would lead to the generation of Th17 cells from Th1 precursor [67]. Furthermore, it has been demonstrated that RA-RARα signalling is required for the expression and activation of STAT4, which triggers the RA-dependent differentiation and functions of Th1 cells.

In addition to Th1 cells, the role of RA has been demonstrated in the regulation of Th17 cell differentiation and functions. Th17 cells are the third subset of Th cells crucial for elimination of extracellular pathogens especially fungal pathogens [70]. In addition to clearing infections, Th17 cells are also important in maintaining barrier functions as well as required to induce tissue inflammation in organ-specific autoimmune diseases [70]. Th17 cells produce effector cytokines IL-17A, IL-17F, and IL-22 and are induced from the naïve T cells in the presence of TGF-β1 and IL-6 [70]. The dichotomy of Tregs and Th17 cells are reciprocally regulated; TGF-β1 alone induced the generation of Foxp3$^+$ iTregs, while IL-6 suppressed the TGF-β1-mediated induction of Foxp3 and induced Th17 cell differentiation. The differentiation of Th17 cells is promoted by the expression of RORγt, a master transcription factor for Th17 cell development [70].

The effect of RA has been extensively studied on Th17 cell differentiation. It has been demonstrated that RA promotes and suppresses the differentiation of Th17 cells at different physiological conditions (Fig. 3.3). RA in combination with IL-6 and TGF-β1 would promote the generation of Foxp3$^+$ iTregs while suppressing Th17 cell differentiation [63, 69, 71]. RA can also inhibit the expression of both IL-6R and IL-23R on Th17 cells, thereby controlling the differentiation of Th17 cells [62]. The inhibitory effect of RA on Th17 cells was found to be mediated by IL-2. Neutralization of IL-2 restored Th17 cell generation even in the presence of RA. IL-2 is a common gamma chain receptor cytokine shown to suppress Th17 cell development via the activation of STAT5. IL-2-, IL-2R-, and STAT5-deficient animals were found to harbour increased frequency of Th17 cells [63]. Consistently, RA-mediated IL-2 production sustains Foxp3 expression while suppressing STAT3 activation, together preventing Th17 cell development [63].

Although RA was shown to suppress Th17 cell development and promote Foxp3$^+$ iTreg generation, lower dose of RA (<10 nM) favours Th17 cell differentiation [63]. VAD diminished Th17 cell differentiation in the small intestine. Lacking intestinal Th17 cells was found to be associated with altered microbiota in VAD mice [72]. Moreover, the generation of Th17 cells within the gut has been found to be associated with segmented filamentous bacteria (SFB). Interestingly, VAD mice were found to have lower numbers of SFB [72]. In addition to its role in Th17 cell generation, RA has also been implicated in inducing gut tropism on Th17 cells. VAD mice revealed lower expression of gut-homing markers (CCR9 and $\alpha^4\beta^7$) on Th17 cells [27]. Taken together these studies demonstrate that RA induces tissue-specific tropism and facilitates in inducing the origin, trafficking, and functions of Th17 cells in the gut.

In addition to Th1 and Th17 cells, RA was found to modulate the induction of Th9 cells. Th9 cells are a distinct subset of Th cells, which predominantly produce IL-9 without producing cytokines of other Th lineages. Activation of naïve T cells in the presence of TGF-β1 plus IL-4 induces the differentiation of Th9 cells. Th9 cells are critical in eliminating extracellular pathogens as well as required to promote allergic inflammation in asthma. In addition, Th9 cells promote potent anti-tumour immune response against solid tumours. Recent findings suggest the inhib-

itory role of RA in suppressing the differentiation of human Th9 cell [73]. RA primed DCs cultured *in vitro* in the presence of TGF-β1 and IL-4 and decreased IL-9 production by Th9 cells while inducing Th1/Th17 cell polarization in humans. However, the exact mechanism through which RA interferes with Th9 cell development is not known yet.

In contrast to the inhibitory effect of RA on Th9 cell differentiation, RA has been shown to promote Th2 cell development. RA induces GATA3 expression, a master transcription factor for Th2 cells, and IL-4 production via the stimulation of RAR pathway enhancing Th2 response. During Th2 differentiation, RA inhibits the production of IFN-γ and IL-12 by suppressing the expression of T-bet while inducing the production of IL-10 and IL-4, together promoting Th2 cell differentiation. The role of RA in potentiating Th2 response was further demonstrated in VAD mice infected with *Trichinella spiralis.* Th2 response was impaired in VAD mice infected with *Trichinella spiralis*. It could be overcome by using RA. Therefore the production of Th2-associated cytokines was increased and pathogen elimination was facilitated [74]. These lines of evidence clearly suggest an important role of RA in the regulation of T-cell differentiation and functions. Further investigation is required to understand the detailed molecular mechanisms by which RA can control the development of distinct T-cell subsets and cytokine production and immune responses of respective T-cell lineages.

Effect of Vitamin A on Innate Lymphoid Cells, NK Cells, and γδ T Cells

In addition to DCs, macrophages, and T cells, VA and its metabolite, RA, influence other immune cells such as innate lymphoid cells, NK cells, and γδ T cells. Innate lymphoid cells (ILCs), the members of the lymphoid cell lineage arising from haematopoietic stem cells, play an important role in maintaining homeostasis and mucosal immunity. ILCs are found in various locations in mucosal tissues such as LP, PP, and MLNs. ILCs have been implicated in linking innate and adaptive immunity, as they have both innate and adaptive functions. Similar to Th subsets, ILCs are primarily divided into three subsets: ILC1, ILC2, and ILC3. ILC1 cells are similar to Th1 cells expressing T-bet and produce IFN-γ and TNF-α providing protection against intracellular pathogens, while ILC2, similar to Th2 cells, specifically express GATA3 and produce IL-4, IL-5, and IL-13, contributing to immunity to helminths as well as allergic inflammation in asthma. ILC3 cells produce IL-17, IL-22, and GM-CSF and express RORγt providing protection against extracellular pathogens [75]. The fate of ILC lineage commitment is decided by the ILC-committed progenitor cells in the bone marrow. Like all antigen-presenting cells, ILCs express MHC-II and thus play a role in antigen processing and presentation.

VA regulates the development, migration, and function of ILCs. Particularly, it has been shown to maintain the balance between ILC2s and ILC3s, thereby protecting against potential immunopathologies. MLN DC-derived RA regulates the migration of ILCs to the intestine by upregulating the expression of CCR9 and $\alpha_4\beta_7$ and downregulating CCR7 expression in ILC1 and ILC3 [76]. RA in the presence of IL-23 and IL-1β significantly increases the production of IL-22 by accelerating the conversion of ILC1 to ILC3, which maintains intestinal homeostasis by the synthesis of antimicrobial peptides such as RegIII and defensins [77]. In contrast, VA negatively regulates ILC2 differentiation by downregulating the expression of IL-7 receptors and subsequently reduced production of IL-13 [78]. The contrasting effect of VA has been shown on ILC2 and ILC3. VAD would enhance ILC2 response as reflected in the increased production of type 2 cytokines such as IL-13, thereby providing resistance to helminth infections, where VAD leads to the suppression of ILC3 proliferation and function.

Natural killer (NK) cells, which are also classified in ILC class, provide protection against tumours and viral infections via cell-mediated immunity. VA appears crucial for NK cell lytic activity and IFN-γ production [79]. VA activates

NK cells by upregulating the expression of RA-inducible genes RAE-1 and MICA/B, which then bind to NKG2D in tumour cells [80, 81]. Also, RA promotes the activation of natural killer T (NKT) cells by increasing the expression of CD1d, which is a lipid antigen-presenting molecule, on DCs [82]. VAD has been shown to impair NK cell activity and have deleterious effects on NK cell differentiation and function.

The effect of VA and its metabolite, RA, was also demonstrated on γδ T cells. RA was shown to induce gut-homing receptor $\alpha_4\beta_7$ integrin and its ligand addressin (MAdCAM-1) on circulating γδ T cells while downregulating the expression of skin-homing receptors. RA would modulate γδ T-cell function by decreasing IL-17A production while enhancing Th17 cell function as reflected in the increased production of IL-22 [83]. RA increases IL-22 production in γδ T cells, which play a crucial role in tissue repair and mucosal immunity. In contrast, RA decreases IL-22 production by inducing IL-22-binding protein in DCs, which capture and inactivate IL-22 cytokine. Also, RA decreases the expression of activation markers, CD25 and CD69, on γδ T cells in a mouse model of experimental autoimmune uveitis [84]. Taken together, VA is critical for regulating the differentiation and function of ILCs, NK cells, and γδ T cells, thereby playing an important role in maintaining immune homeostasis.

Effect of Vitamin A on the Development of Mucosal IgA$^+$ B Cells and Functions

B cells are the primary cell types of humoral immunity that produce antibody. Upon activation, naïve B cells differentiate into plasma cells, which produce high-affinity antibodies and lead to the generation of memory B cells. RA, similar to its effect on DCs, T cells, and ILCs, induces the expression of gut-homing receptors on B cells. DCs present in MLN, LP, and PP of the intestine drive the differentiation of naïve B cells into immunoglobulin A (IgA)-secreting B cells and thus contribute to mucosal immunity. Several evidences indicate that RA produced by GALT DCs and intestinal stromal cells mediates IgA class switching in activated B cells and is crucial for the generation of IgA$^+$ B cells, as administration of RAR antagonist LE540 significantly decreased IgA production [85]. VAD mice showed reduced number of B cells in the mucosa due to loss of IgA-producing cells. In addition to RA, other cofactors which play an important role in the development of IgA$^+$ B cells include cytokines such as TGF-β1, IL-5, and IL-6 induced by microbial stimulus. Moreover, in the presence of RA and TLR-mediated activation of MYD88 signalling, follicular dendritic cells and bone marrow-derived DCs induce TGF-β1 secretion via inhibition of SOCS3, thereby enhancing IgA production from B cells [86]. In addition, RA increases the expression of the transcription factor NFATc1 which increases IgA production by B1-B cells activated during early response to pathogens and maintains mucosal integrity during homeostasis [87]. Therefore, VAD leads to reduced production of IgA by B cells in the intestine, indicating the importance of RA in regulating IgA responses and humoral immunity.

In general, B cells play an important role in inflammatory responses. However regulatory B cells (Bregs) are a particular subset of B cells with immunosuppressive effects that maintain tolerance. Bregs produce IL-10, which plays a crucial role in the suppression of T-cell proliferation [88]. RA produced by DCs promotes the development of Breg cells which produces IL-10 [88]. There are emerging reports suggesting that RA negatively regulates T-cell-activated B-cell development and proliferation through downregulation of NF-κB signalling and decreased expression of cell cycle regulatory factors leading to cell cycle arrest [89].

Vitamin A and Diseases

Role of Vitamin A in Inflammatory Diseases

Chronic inflammatory diseases occur as a consequence imbalance between effector and regulatory mechanisms of immune system. Several

factors such as genetic polymorphisms, environmental factors, and dietary components correlate with the imbalance of immune homeostasis, which may lead to inflammatory and autoimmune diseases. Genetic and environmental factors together with enhanced immune response to self-antigens cause autoimmune diseases. VA plays a determining role in the outcome of chronic inflammatory disorders and autoimmunity since it regulates the differentiation and functions of various immune cells. Here, we discuss the role of VA in the manifestations of autoimmune diseases, as to how RA metabolism can be modulated and exploited for successful immune intervention.

IBD is a chronic inflammation of the gastrointestinal tract resulting from an abnormal immune response against oral antigens. IBD is comprised of Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by severe inflammation of the small and large intestines, respectively. Effector T cells such as Th1/Th17 cells play a crucial role in inducing disease pathogenesis in IBD. More precisely, an imbalance between effector (Th1/Th17) cells and Treg cells leads to tissue inflammation in IBD. VA has been implicated in IBD. UC patients show increased expression of CYP26A1 and decreased expression of ALDH1a1 as well as reduced levels of RA. Both CYP26A1 and ALDH1a1 enzymes are crucial to maintain physiologic concentration of RA. In a mouse model of acute colitis, DSS (dextran sulphate sodium) leads to reduced expression of β_7 integrins on DCs, affecting the migration of DCs to the intestine and production of RA, together resulting in intestinal inflammation. Administration of RA suppressed DSS-induced colitis, suggesting the protective role of VA in IBD [90]. Moreover, administration of RA to DSS-treated mice was shown to increase Foxp3 expression while simultaneously inhibiting IL-17 via activation of RARα, thus restoring the balance between Th17 and Tregs in UC [90]. Furthermore, RA inhibited the activation of NF-κB and reduced the production of pro-inflammatory cytokines such as TNF-α, thereby diminishing intestinal inflammation [90]. Consistently, VAD mice showed increased susceptibility to UC. RA has been shown to enhance IL-22 production from γδ T cells and ILC3, which, in turn, promotes tissue repair and barrier functions improving remission in UC patients [84]. In contrast, high levels of RA due to the polymorphism in *CYP26B1* and increased expression of ALDH1a enhance the risk of CD, suggesting a completely opposite role for RA in CD [91]. It has been reported that CYP26B1-deficient T cells had increased levels of RA and decreased levels of IL-17 and TNF-α, leading to attenuation of inflammation and disease progression in the colon. However, the expression of Foxp3 and gut-homing receptors such as $\alpha_4\beta_7$ integrin and CCR9 remained unaffected in CYP26B1-deficient T cells [91]. The more precise molecular role of VA in IBD has remained to be elucidated with further investigation.

The roles of RA and vitamin A were also investigated in multiple sclerosis (MS). MS is a chronic autoimmune and demyelinating disease of the central nervous system (CNS) mediated by autoreactive T cells, B cells, and monocytes against CNS proteins. VA inhibits the production of IL-1β, IL-12, TNF-α, and nitric oxide in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. VA supplementation induces TGF-β1 production and Foxp3 expression while downregulating RORγt expression and therefore IL-17 levels in MS patients [92]. Th17 cells are the primary effector T-cell population involved in the development of EAE. RA has been shown to suppress the development and functions of Th17 cells while increasing the frequency of $Foxp3^+$ Tregs in EAE [92]. Similarly, VAD contributes to the development of tissue inflammation in MS due to disruption of immune tolerance and therefore causes the imbalance of T-cell subsets.

A role for RA was also suggested in psoriasis. Psoriasis is a chronic inflammatory disease of the skin characterized by abnormal proliferation of keratinocytes due to uncontrolled differentiation of DCs, macrophages, and T cells. Under normal homeostasis, RA stimulates the proliferation of keratinocytes. In psoriasis, RA displays anti-proliferative effect on keratinocytes [93]. Treatment of psoriatic patients with

RA improved disease severity by downregulating the expression of migration inhibitory factor-related protein-8, thereby inhibiting the migration of activated lymphocytes to the skin and therefore controlling the hyperproliferation of keratinocytes. DCs resident in the skin express ALDH1A and produce RA to induce Foxp3 expression and promote Treg differentiation in the epidermis of the skin [94]. CYP26A1 is highly expressed in epidermal keratinocytes in psoriatic patients, leading to RA degradation and therefore contributing to disease progression [95]. Administration of pharmacological inhibitors of CYP26A1 and retinoids to psoriatic patients has been clinically successful in improving the disease status of the patients without any side effects [95]. Cytokines produced by Th17 cells, e.g. IL-17 and IL-22, appeared to be crucial in inducing tissue inflammation in psoriasis. Neutralization of IL-17 demonstrated beneficial effects on tissue inflammation in psoriasis, and anti-IL-17 antibody therapy has become a first-line therapy for psoriasis. It has previously been discussed in this chapter that VA and its metabolite, RA, suppress Th17 cell development and therefore might be useful in the treatment of psoriasis.

The role of RA has also been implicated in other inflammatory diseases such as systemic lupus erythematosus (SLE) and atherosclerosis. SLE is a systemic autoimmune disorder characterized by the production of autoantibodies against several self-antigens. Patients with SLE have increased frequency of Th17 cells and reduced levels of VA. However, in a mouse model of SLE, treatment with RA did not induce Tregs, but it did create a pro-inflammatory microenvironment. Therefore, RA might be having an adverse effect on SLE [96]. Atherosclerosis is a chronic inflammation of blood vessels characterized by excessive lipid accumulation and coagulation. Treatment with RA promotes fibrinolysis, reduces thrombus formation, and inhibits the expression of vasoconstrictor thromboxane A2 in vascular smooth muscle cells [97]. CYP26B1 is constitutively expressed in human vascular smooth cells and is upregulated in the macrophages of atherosclerotic patients, reducing the levels of RA [98]. Administration of RA in combination with talarozole increased cellular RA concentrations which decreased cell proliferation in atherosclerotic patients and improved the clinical condition.

Type I diabetes mellitus is an autoimmune disease in which both $CD4^+$ and $CD8^+$ effector T cells are involved in the generation of an inflammatory immune response against antigens present on the surface of pancreatic β cells. VA has been suggested to play an important role in the maintenance of glucose homeostasis by regulating the release of insulin and glucagon [99]. VA has an immunosuppressive role in diabetes by inhibiting the production of pro-inflammatory cytokines such as IFN-γ, IL-17, and TNF-α by Th1 and Th17 while promoting $Foxp3^+$ Treg differentiation. VA reduces the oxidative damage of β cells in the islets and decreases the migration and infiltration of immune cells into the islets by downregulating the expression of chemokines. VAD mice showed impairment in insulin secretion, resulting in the destruction of pancreatic β cells and increased digestion of the pancreatic tissue with collagenase. Therefore, VA and its derivative, RA, would induce immune tolerance in patients with diabetes as a result of its autoimmune- protective effects by inhibiting both $CD4^+$ and $CD8^+$ IFN-γ-producing effector T cells and enhancing $Foxp3^+$ Tregs [100].

Vitamin A and Infection

VAD leads to dysregulated immune responses in infections. As described earlier in this chapter, RARα-deficient mice infected with *Listeria monocytogenes* showed impairment in the development and functions of both $CD4^+$ and $CD8^+$ T cells, implicating that RA plays a crucial role in mounting effective immune response against pathogens [101]. RA also plays an important role in infection with other intracellular pathogens such as *Mycobacterium tuberculosis*, as administration of RA in *Mycobacterium tuberculosis*-infected mice enhanced macrophage activation and production of pro-inflammatory cytokines, resulting in killing of the pathogen [102]. Similarly, infection with *Toxoplasma gondii* in VAD mice resulted in impaired protective Th1 response

due to reduced production of IFN-γ facilitating the survival of pathogen inside the host [103]. In addition to its direct effect on eliminating intracellular pathogens, VA was also shown to have an impact on the efficacy of vaccination. Vaccination with *E. coli*-derived heat-labile enterotoxin in VAD mice resulted in diminished Th1 and Th17 response against intestinal commensal microflora [104]. Thus, VA acts as an adjuvant since vitamin A supplementation aids in increasing the potency of vaccination by enhancing T-cell responses.

Conclusions

VA and its derivative, RA, are an integral part of the normal development and functioning of the immune system. The physiological functions of RA in the immune system are determined by several factors in the microenvironment such as VA content, cytokine milieu, TLR signalling, gut microbiota-derived fatty acids, GM-CSF, prostaglandin, and others. RA binds to its nuclear receptors and regulates the expression of multiple genes which profoundly affect the development and function of various immune cells such as DCs, macrophages, ILCs, T and B cells, etc., in the mucosal and peripheral tissues. Based on the published literature, it is postulated that RA has a dual role: (1) under normal homeostasis, RA promotes the differentiation and function of regulatory T cells and induces immune tolerance against varieties of antigens during inflammation and infection, and (2) in the presence of pro-inflammatory cytokines, RA promotes the effector T-cell responses. VAD increases susceptibility to infections and autoimmune diseases as a result of dysregulated immune responses. VA supplementation overcomes the defect in immune response in VAD individuals and facilitates in mounting effective mucosal and systemic immune responses against infections and during vaccination.

VA plays an immunomodulatory role in the context of infections and inflammation, and therefore RA can be exploited as an effective therapeutic strategy for the treatment of infections, cancer, and chronic inflammation in human diseases. Moreover, RA acts as an adjuvant for successful vaccination by enhancing vaccine-induced immunity. However, the variable outcomes of RA treatment for successful immunotherapy depend upon the immunological status of the patient and the local microenvironment. Despite the fact that VA has a crucial role in regulating immune responses, the use of RA as a successful strategy for immunotherapy in immunocompromised patient would remain obscure due to adverse side effects and toxicity associated with RA treatment. Therefore, further studies are needed to understand the mechanistic insights of immunomodulation by VA.

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Vitamin K and the Immune System

4

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Contents

Key Points

- Vitamin K can act as a cofactor for some plasma proteins, thereby affecting immune and inflammatory responses particularly mediated by T cells.
- Vitamin K can inhibit cell growth by promoting apoptosis and autophagy.
- Low levels of vitamin K have been associated with inflammatory diseases and some types of cancer.

Introduction

Vitamin K is one of the fat-soluble vitamins with two natural types: phylloquinone (K1) and menaquinone (K2, or MK-n). Vitamin K1 is synthesized in plants; however, vitamin K2 is mostly produced

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by microorganisms including intestinal flora in the lower intestine [1]. Menaquinone can help the maintenance of vitamin K in the host. Nevertheless, it is not clear whether it takes part in nutritional status of vitamin K or not [1]. The synthetic form of vitamin K named menadione (vitamin K3) is alkylated in the liver and produces menaquinones [2] (for more information on chemical structures of the three types of vitamin K, see [3]).

When vitamin K is absorbed from the intestine, triglyceride-rich lipoprotein chylomicrons transport vitamin K to the liver and activate K-dependent proteins [2]. Vitamin K1 is absorbed via an energy-dependent process in the small intestine, while vitamins K2 and K3 are absorbed by passive diffusion in both the small intestine and the colon. In general, its absorption requires bile salts, pancreatic juices, and minimum dietary fat. Many tissues particularly in cellular membranes contain the low levels of vitamers K (in a mixture) [1].

Vitamin K is mainly found in green leafy vegetables. Spinach, broccoli, and asparagus are the main sources of vitamin K in vegetable group. Additionally, plant oils can be a good source of phylloquinone [2].

Vitamin K plays key roles in different physiological functions such as blood coagulation, bone metabolism [1], and the regulation of some enzyme systems. It also can act as a cofactor of some plasma proteins [2, 4] and affect immune parameters, particularly T cells and inflammatory pathways [4–7]. However, there is scarce information about how the immune system will be influenced by vitamin K.

Vitamin K and the Immune System

The innate immune system is capable of protecting against invading pathogens with no earlier exposure and immunization. There are limited studies on the effects of vitamin K on the innate immune system [8]. However, one of the main components of the innate immune system is the complement system that contains over 30 proteins. The complement system can improve the adaptive immunity as well. It is stimulated through three main pathways including classical, alternative, and lectin pathways. Vitamin K is a crucial factor for the modification of protein S. Protein S is suggested to be related to C4B-binding protein (C4BP), a potential soluble inhibitor of the classical and lectin pathways of complement. C4BP can act as a survival factor for B cells through interaction with CD40. Recently, computer-based molecular analysis and recombinant DNA technologies demonstrated that the structure of C4BP could influence upon its function. In this manner, vitamin K can indirectly affect immunity [9].

Additionally, it has been reported that derivatives of vitamin K (vitamin K3 and vitamin K5) can inhibit the proliferative response and cytokine production by activated T cells [6, 4]. Helper T (Th) cells secrete cytokines and chemokines. Disruption of the balance between Th1 and Th2 cytokine production can lead to the development of autoimmune diseases. Little is understood about the effects of vitamin K derivatives on cytokine production. However, it has been shown that vitamin K derivatives have a suppressive effect on the production of tumor necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), IL-6, and IL-10. They can also increase the frequency of CD4+, CD25+, and Foxp3+ regulatory T (Treg) cells [6].

Recent studies indicated that cellular redox status might play a crucial role in immune functions. An in vitro study revealed that the treatment of lymphocytes with vitamin K3 decreased glutathione to oxidized glutathione ratio (GSH/GSSG) and increased the levels of reactive oxygen species (ROS). Based on the mass spectrometric analysis, menadione directly can interact with thiol antioxidant GSH and suppress extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and nuclear factor-κB (NF-κB) in lymphocytes [4]. Due to the anti-oxidative properties of vitamin K3, NF-κB expression can be suppressed. NF-κB, a central transcription factor, influences the expression of different cytokines and enzymes involved in immune responses [4]. Of note, vitamin K3 can inhibit the activation of NF-κB more than natural forms of vitamin K. However, anti-inflammatory effects have been reported with both vitamins K1 and K2 [3].

Vitamin K and Inflammation

Rats receiving vitamin K-deficient diet showed an increase in the expression of genes involved in inflammatory response. In vitro studies also showed a reduction in the production of inflammatory parameters including IL-6 in human macrophages and fibroblast cells treated with vitamin K. In contrast, lipopolysaccharide (LPS)-induced immune responses were suppressed in rats which consumed sufficient vitamin K1 [1]. In addition, dietary supplementation with vitamin K1 can suppress an inflammation induced by lipopolysaccharide in rats [1].

In the Framingham Offspring Study, an inverse link was found between vitamin K status (measured by plasma phylloquinone level and phylloquinone intake) and the overall inflammation index including IL-6 and C-reactive protein (CRP) levels [7].

A prospective study reported an inverse association between plasma phylloquinone levels and serum levels of IL-6 and CRP. Of note, cytokine concentrations remained unchanged after 3 years of supplementation with phylloquinone [10].

The Role of Vitamin K in Inflammatory Diseases

Inflammatory bowel disease (IBD) is a chronic inflammatory disease affecting the gastrointestinal tract. It has been found a link between low levels of vitamin K and Crohn's disease (a type of IBD). However, the precise mechanism through which vitamin K may be protective against intestinal inflammation remained unclear [11].

Acute respiratory distress syndrome (ARDS) is accompanied by the production of inflammatory mediators including cytokines, adhesion molecules, chemokines, and bioactive lipid products from immune cells. Based on the study by Tanaka et al., vitamin K3 reduced lipopolysaccharide-induced acute lung injury via inhibition of nuclear factor-κB activation. Accordingly, vitamin K3 may be an effective treatment for acute lung injury. However, further studies are needed to confirm its efficacy [3].

Vitamin K and Cancer

In recent years, accumulating studies have reported antiproliferative effects of vitamin K (K1, K2, and K3) on cancer cells [5, 12]. Vitamin K2 has shown anti-tumorigenic effects (growth suppression and apoptosis) on cancers involving the lungs, ovaries, liver, prostate, and bone marrow [13–16]. Vitamin K can, thus, be considered in the treatment of cancers.

Vitamin K2 can block the cell cycle at the G1 phase and induce the caspase-3-mediated apoptosis [5]. A cell line study revealed that vitamin K2 can induce apoptosis of bladder cancer cells via breaking DNA. It suppressed cell growth, lowered the viability of bladder cells, activated caspase-3, and fragmented poly ADP-ribose polymerase (PARP) [5].

Another pivotal pathway that regulates apoptosis is related to mitochondria. Therefore, targeting the mitochondria is likely to be a novel strategy for the treatment of cancer [17–19]. Vitamin K2 can also induce mitochondria-related apoptosis in bladder cancer [5].

Mitochondria-mediated apoptosis mainly occurs following the dysfunction of mitochondria, which can be induced by the loss of mitochondrial membrane potential and apoptotic factors [5]. They activate caspase cascade. Caspases are a family of aspartic acid-specific proteases that play a role in apoptosis [20].

In addition, the upregulation of Bax, Bak, and Puma (proapoptotic proteins of Bcl-2 family) can directly or indirectly lead to the loss of mitochondrial membrane potential. According to the study by Satoki et al., vitamin K2 can induce the expression of Bax and Bak in Hela cancer cells [21].

There are several human model studies on the anticancer effects of vitamin K. For instance, Tamori et al. showed that vitamin K2 can prevent hepatocarcinogenesis in patients with hepatic cirrhosis [22]. Two meta-analyses of randomized controlled clinical trials also revealed that analogs of vitamin K prolonged disease-free survival [23, 24]. Based on another meta-analysis, an analog of vitamin K2 can prevent the formation of secondary tumors in the liver tissue and enhance the survival rate in patients with hepatocellular

carcinoma. Suggested mechanisms included the activation of apoptotic pathways and inhibition of NF-κB [25].

Autophagy is a physiological strategy of the cell to remove dysfunctional components or long-lived cytosolic proteins. In autophagy, proteins and organs in the cytosol are degraded via lysosomes. Autophagy-defective cells are at risk for metabolic stress, genomic damage, and tumorigenesis. Therefore, autophagy plays a role in preventing cancer [12]. Interestingly, vitamin K can inhibit cell growth through both apoptosis and autophagy in leukemia and colon cancer cells [26].

Conclusions

Recent studies have shown the role of vitamin K in the regulation of immune responses and low levels of the vitamin linked to inflammatory diseases and some types of cancer. However, more studies are necessary for making a decision regarding the effects of vitamin K on the immune system and its possible mechanisms of action.

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Vitamin C and the Immune System

5

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Key Points

- Vitamin C orchestrates the function of the human immune system by supporting various aspects of both the innate and adaptive immune system including epithelial barrier function, chemotaxis and antimicrobial activities of phagocyte cells, natural killer (NK) cell functions, and lymphocyte proliferation and differentiation.
- Severe vitamin C deficiency has been associated with impairments in immunity and increased susceptibility to more infections, while vitamin C supplemenvtation seems helpful to prevent and treat infections.
- Supplementation with vitamin C, especially in groups such as the elderly, has been shown to reduce the duration and severity of cold symptoms by enhancing various immune cell functions.
- Ensuring sufficient vitamin C levels may be particularly an essential factor in conditions involving an additional challenge in the immune system such as inflammation, autoimmunity, and cancer.

Introduction

Immunity and Nutrition

The immune system is a well-evolved web of cells and proteins that sense foreign substances and protect the host from invading pathogens such as bacteria, viruses, fungi, and parasites [1–3]. In addition to genetic factors, numerous environmental factors can influence immune system functions. One of those is nutrition [4, 5]. Mechanisms linking the immunity and nutrition are still inadequately understood [6, 7]. It is, however, a well-known fact that immune cells, similar to other cells in the body, need an adequate supply of energy, macronutrients, and micronutrients at the proper times and amounts to ensure appropriate development, maintenance, and function [2].

In relatively few years, "immunonutrition" has become an interesting subject to discover and research, and most papers on immunonutrition investigate its influence on a wide range of important clinical conditions [8, 9]. According to the concept, immunonutrients are nutritional elements, which affect the inflammatory and immune response [10, 11]. Besides macronutrients (free fatty acids, glucose, nucleotides, and amino acids), growing evidence reveals that a sufficient amount of micronutrients including trace elements and vitamins plays an important part in maintaining immune function and could, therefore, help disease prevention and health promotion [2].

Among the essential micronutrients, vitamin C plays a central role in the regulation of normal immune function [12]. There is a big history behind the discovery and research on vitamin C [13]. One of the greatest efforts that helped to establish the framework of research on vitamin C was the effect made by a brilliant Hungarian-born researcher named Albert Szent-Györgyi. His notable discoveries helped to identify the role of vitamin C in immune functioning. Subsequently, he was awarded the 1937 Nobel Prize in Physiology or Medicine [13, 14].

Vitamin C and the Body

Vitamin C, also known as ascorbic acid, is a water-soluble micronutrient. Most mammals use the enzyme L-gulono-γ-lactone oxidase to synthesize vitamin C in the liver. But humans and many primates lack the ability to synthesize vitamin C due to carrying a version of the GULO pseudogene and therefore require an adequate and regular dietary intake of vitamin C [15–19]. cDNA sequence analysis of the human GULO gene showed a remarkable accumulation of many mutations making the gene inactive. There are many controversies surrounding the cause of these genetic mutations. Among which are environmental factors including radiation exposure [20] and viral infections [21].

Guinea pigs, like humans, also possess a GULO pseudogene in the genome with premature stop codons. Therefore, they are a good model to investigate the effects of vitamin C deficiency [22]. GULO knockout (GULO−/−) and a senescence marker protein-30 knockout (*SMP30KO*) mouse models with the blocked or altered biosynthesis of vitamin C in the liver are other available options [22–24].

The normal serum range for vitamin C is considered to be 30–90 mol/l (0.5 and 1.6 mg/dL). Serum levels between 11 and 23 mol/l show marginal vitamin deficiency, and amounts below 11 mol/l (<0.18 mg/dl) indicate deficiency [25]. The Recommended Dietary Allowance (RDA) of vitamin C for human adults was defined as a set of reference values by the Food and Nutrition Board (FNB) in 1943. The current recommendation for average daily level of intake of vitamin C (according to the RDA) is about 90 mg/day for men and 75 mg/day for women [26, 27]. Daily intake of 100 mg per day of the vitamin appears to be adequate to saturate vitamin C concentrations in the blood, white blood cells, and other tissues in healthy adults and is associated with decreased rate of mortality from heart diseases, stroke, and cancer [27].

Vitamin C is a cofactor for hydroxylase and monooxygenase enzymes, and the human body utilizes it to synthesize essential compounds like collagen (a type of structural protein that makes up connective tissue and aids in wound healing) and other compounds, such as L-carnitine and neurotransmitters [27]. This monosaccharide also acts as a necessary factor for the enhancement of iron absorption from non-heme iron sources, the transformation of cholesterol to bile acids, and the process of adrenal steroid genesis [8, 27–29]. Ascorbic acid, as a potent antioxidant, is known to neutralize free radicals and other reactive oxygen species (ROS) and reduce the risk of inflammation and susceptibility to diseases [30, 31]. Vitamin C is also protective against deoxyribonucleic acid (DNA) mutations induced by oxidative stress [32, 33]. Current evidence indicates that getting sufficient vitamin C could improve immunity, thereby protecting against medical conditions from a common cold to a more serious issue like cancer [27].

Vitamin C and Immunity

Principal cells and tissues of the immune system require to be fed, and the development, maintenance, and optimal functioning of the immune system need an adequate supply of energy and nutrients [2]. Several studies have demonstrated that there is a good scientific rationale for the role of vitamin C in the regulation of the human immune system [34]. In addition to its antioxidative effects, vitamin C plays a fundamental role in its immune-modulating effects by being a cofactor for several enzymes involved in biosynthetic and gene expression [35]. Recent studies have highlighted the immune-enhancing and immune-modulating effects of vitamin C on both the innate and adaptive wings of the immune system. This micronutrient helps to maintain epithelial barrier integrity and promote NK-cell activity and differentiation of naive CD4+ T cells into helper T (Th) 1 cells (high IFN-γ-producing T cells). In addition, vitamin C has been shown to affect the production of pro-inflammatory cytokines/chemokines and the expression of adhesive molecules [2, 36].

All of the white blood cells would be influenced by vitamin C. There are three main subsets of lymphocytes: T cells, B cells, and NK cells [37]. Vitamin C supplementation can improve immune responses mediated by different subsets of lymphocytes, such as antimicrobial and NK-cell activities, lymphocyte proliferation, and delayed-type hypersensitivity (DTH) response [38–43]. In addition, healthy adults receiving 1 g/day of ascorbic acid over 28 days revealed an increase in T-lymphocyte proliferation and cytokine and immunoglobulin production in response to infection [44]. Diets deficient in vitamin C (5–20 mg/day) decreased DTH responses to several antigens, while receiving 60–250 mg vitamin C daily normalized DTH responses [38]. Moreover, vitamin C supplementation (500 mg/day for 1 month) in older people known to have low levels of vitamin C could enhance T-cell proliferative response [40].

Activated phagocytic cells such as neutrophils and macrophages contain high levels of vitamin C. Vitamin C stimulates migration of

phagocytic cells including neutrophils and monocytes to the site of infection and enhances phagocytosis, oxidant generation, and microbial killing [45, 46]. Vitamin C supplements (1–3 g/day, over 1 week for each dose) have been shown to enhance neutrophil chemotaxis in healthy adults [44]. Additionally, it can prevent inappropriate or excessive activation of the immune system as well as inhibit tissue damage by increasing neutrophil apoptosis and clearance by macrophages and decreasing neutrophil necrosis and NETosis. In other words, vitamin C is necessary for the immune system to promote an adequate response against invading pathogens without producing excessive damage to the host tissues [35].

Vitamin C is essential to the functioning of immune cells [47]. White blood cells became saturated at a dose of 100 mg of vitamin C [48]. It is important to point out that data concerning plasma concentrations do not necessarily indicate that all tissues would be saturated at this dose. White blood cells actively accumulate vitamin C against a concentration gradient, and vitamin C levels in these cells are 50- to 100-fold higher than in plasma [49], which may indicate functional roles of the vitamin in the principal cells of the immune system [50, 51]. Leukocytes, such as neutrophils, monocytes, and lymphocytes, acquire vitamin C via sodium-dependent vitamin C transporters (SVCT) and sodium-independent glucose transporters (GLUT) [45]. Potential antioxidant properties of vitamin C allow the immune cells to be protected from detrimental ROS produced in the inflammatory response [52].

Here we review the literature on the supportive effects of vitamin C on different immune cells. In particular, it describes the vital involvement of vitamin C in the regulation of the immune system to prevent tissue damage and discusses how low levels of vitamin C may weaken the immune system. Finally, the chapter ends with a short discussion of the relevance of this essential nutrient for situations known to challenge the immune system such as the aging process, infectious diseases, and other immune-related disorders.

Vitamin C and the Immune Cells

Vitamin C and Epithelial Barrier Function

Vitamin C is well known for its immune-enhancing effects. Vitamin C as an enzymatic cofactor plays an essential role in the maintenance of gut barrier function and the formation of collagen in the skin [53]. Optimal levels of vitamin C stimulate fibroblast migration, matrix deposition, and neovascularization in the process of wound healing by modulating levels of transcription factors such as HO-1, TGFβ, CTGF, and VEGF. In this manner, vitamin C helps in wound healing [54].

Leukocytes

Leukocytes, such as neutrophils and monocytes, accumulate maximum vitamin C concentrations at dietary intakes of ~100 mg a day [40]. Indeed, the accumulation of vitamin C points to the significant functions of these cells. Vitamin C is particularly concentrated in leukocytes and it is used quickly during infection [48, 53]. It is thought that the accumulation of millimolar concentrations of vitamin C into neutrophils would help the activation of oxidative burst and keep the cells safe from oxidative damage [55]. Disruption of the balance between oxidant generation and antioxidant defenses causes changes in multiple signaling molecules, particularly the pro-inflammatory transcription factor (NF-κB) [56]. Oxidants can activate NF-κB, which triggers a signaling cascade resulting in the constant production of oxidative species and inflammatory mediators [57]. Vitamin C would attenuate oxidant generation and also NF-κB activation in dendritic cells and neutrophils isolated from infected GULO-knockout mice [58, 59].

Phagocytic leukocytes secrete non-specific toxins, such as superoxide radicals, hypochlorous acid, and peroxynitrite, in response to invading pathogens. These ROS destroy pathogens as well as have the ability to damage leukocytes [60]. Vitamin C can protect leukocytes from oxidative

stress [61]. Phagocytic leukocytes produce cytokines, such as interferon that has antiviral activities [62]. Vitamin C has been shown to increase interferon levels in vitro [63]. Moreover, it can regenerate the antioxidant form of vitamin E from its oxidized form [64].

Neutrophils

Vitamin C promotes neutrophil migration to the infection site. It also improves phagocytosis, oxidant generation, and microbial killing (Fig. 5.1). Simultaneously, it keeps the host tissue safe from excessive damage through the enhancement of neutrophil apoptosis, clearance using macrophages, and decreasing the neutrophil necrosis and NETosis. Therefore, vitamin C is essential to the immune system. In this manner, it can mount a suitable immune response against pathogens, and at the same time, it can prevent excessive damage to the host.

Neutrophils absorb invading pathogens and then kill absorbed pathogens using strong bursts of short-lived oxygen free radicals. It seems that ascorbic acid plays a role in some neutrophil functions such that it increases chemotaxis and particulate ingestion, improves lysozyme-mediated non-oxidative killing, protects against the poisonous effects of superoxide anion radical, suppresses the halide peroxide-myeloperoxidase system without a noticeable bactericidal effect, and stimulates the hexose monophosphate pathway [65]. Neutrophils scavenge up the potent oxidation products formed during the process of killing and removing microorganisms, once they have done their work to destroy the bacterial cell. Surprisingly, lower concentrations of the vitamin and abundant production of dangerous oxidizing molecules can impair neutrophil function, and neutrophils would kill themselves [65, 66]. A study indicated that when people have an oral intake of 1000 mg or more of vitamin C, neutrophils can perform more strongly [67].

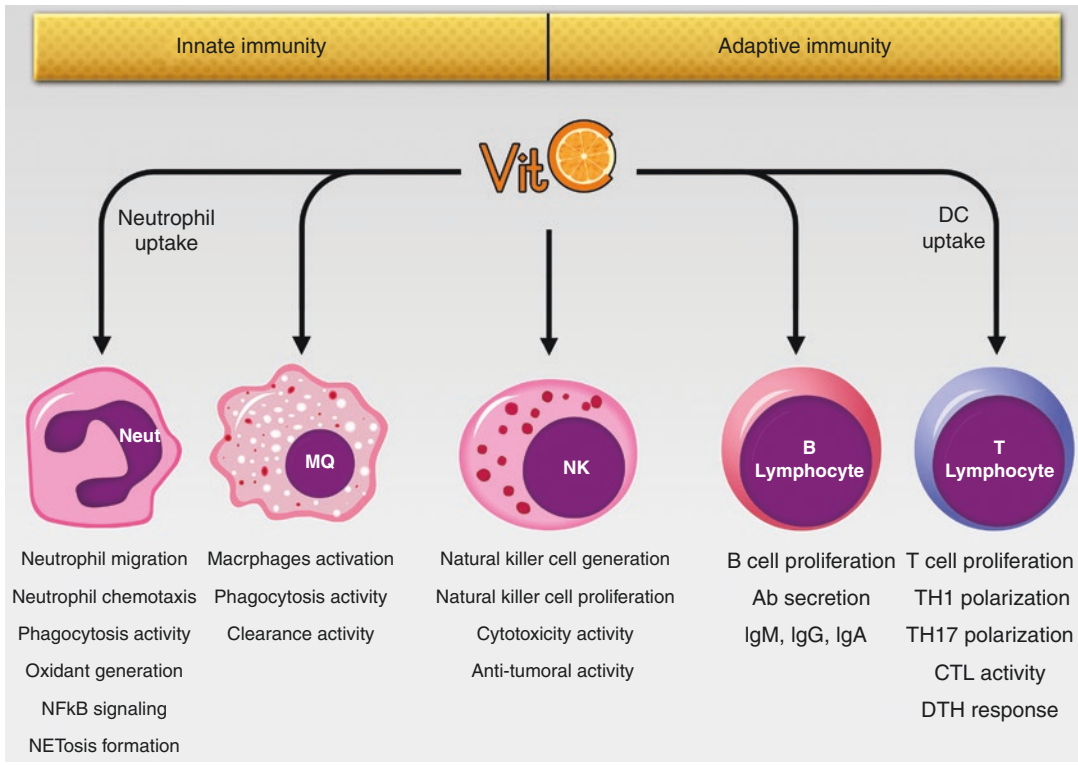

Fig. 5.1 Vitamin C and the immune system

The enhanced function of neutrophils in the presence of adequate vitamin C has been thoroughly proved in a way that clinicians have started to consume vitamin C at 1000 mg per day for patients suffering from chronic granulomatous disease (CGD), a condition in which neutrophils lack a right killing ability as soon as they have ingested bacteria [68]. Same improvements in neutrophil function have been observed in the much larger population of people suffering from asthma, another disease in which neutrophil disability can make the patients' clinical status worse [69]. Also, supplementation with vitamin C-rich SunGold kiwifruit (2 kiwifruit a day, providing about 259 mg of vitamin C per day, for 4 weeks) in 14 young men having suboptimal vitamin C status (<50 μmol/L) at baseline increased vitamin C concentration in plasma and neutrophils, resulting in enhanced neutrophil chemotaxis and oxidant generation [70].

Chemotaxis

Neutrophil migration into infected tissues is considered as an early step in innate immunity. Concerning pathogen- or host-derived inflammatory signals, IL-8, leukotriene B4, and complement component C5a, and neutrophils move to the site of infection. The migration of neutrophils in response to chemical stimuli is called chemotaxis, and the random migration is called chemokinesis. Neutrophils express over 30 various chemokines and chemoattractant receptors by which they are able to sense and quickly react to tissue damage signals [71].

It is thought that impaired leukocyte chemotaxis is partially the result of enhanced levels of anti-inflammatory and immune-suppressive mediators (e.g., IL-4 and IL-10) [72]. It also appears that vitamin C depletion, which is often found in severe infection, might exacerbate the condition. Patients suffering from recurrent infections have compromised neutrophil chemotactic ability, which can be improved in response to supplementation with vitamin C [73–75]. In neonates with suspected sepsis, supplementation with 400 mg of vitamin C per day dramatically improved neutrophil chemotaxis [76]. CGD and Chediak-Higashi syndrome (CHS) are genetic disorders of neutrophil function [77]. Although vitamin C supplementation does not address the underlying genetic defects of these disorders, it may reinforce the function of additional antimicrobial activity in these cells. For instance, leukocyte chemotaxis was improved in patients with CGD after enteral or parenteral supplementation with vitamin C. Also, neutrophils isolated from two children suffering from CHS indicated improved chemotaxis after supplementation with 200–500 mg of vitamin C per day [47]. The neutrophil function can, therefore, be considered as an indicator of vitamin C status [78]. Large doses of vitamin C are able to reduce blood histamine levels. Notably, the more the reduction in histamine levels, the greater the improvement in neutrophil chemotaxis [79].

Phagocytosis and Microbial Killing

Upon infection, neutrophils require vitamin C in order to neutralize extremely high levels of oxidative stress [2, 80]. ROS are produced during the respiratory burst to destroy pathogens and are elevated in the inflammatory response. The oxidant-antioxidant balance is considered as an important determinant of immune function, and due to the higher percentage of polyunsaturated fatty acids in their plasma membranes, immune cells are especially sensitive to alterations of this balance. Oxidative damage can cause losing membrane integrity and altered membrane fluidity which lead to changes in the transmission of signals both within and between various immune cells [81].

By phagocytosing, killing, and digesting bacteria and fungi, neutrophils provide the first line of defense of the innate immune system. Neutrophils generate ROS which can damage the cells themselves and surrounding tissues excessively. The carotenoid fucoxanthin has been analyzed regarding its antioxidant and anti-inflammatory actions. Vitamin C has also powerful antioxidant actions. The results of this study indicate an immunomodulatory impact of the carotenoid fucoxanthin alone or in combination with vitamin C on the function of human neutrophils [82].

Neutrophil Apoptosis and NETosis

Inflammation is considered a useful host reaction to the foreign challenge. It involves numerous soluble factors and cell types, such as polymorphonuclear granulocytes and neutrophils. Programmed cell death (apoptosis) of neutrophils has been reported in vitro as well as in vivo. Apoptosis is believed to be important to resolve inflammation since this process allows for engulfment and removal of senescent cells before their necrotic disintegration [83]. Key effector enzymes in the apoptotic process are caspases. They culminate upon phosphatidylserine exposure and mark the cells for uptake and clearance by macrophages [84].

Vitamin C is thought to protect against caspase-dependent apoptosis after neutrophil activation. In vitro researches have indicated that loading human neutrophils with vitamin C can improve *E. coli*-mediated apoptosis of neutrophils [85]. Studies report that neutrophil apoptosis in patients suffering from severe infection is attenuated compared with control group participants [47]. Immature neutrophils released during serious infection also resist apoptosis and their lifespans are longer [86]. It is found that plasma from septic patients can inhibit apoptosis in healthy neutrophils, arguing that the increased in vivo survival of neutrophils during inflammatory conditions is because of pro-inflammatory cytokines. High dosages of vitamin C have been found to modulate cytokine levels in patients suffering from cancer. It is also found that intravenous supplementation with 450 mg of vitamin C per day in patients who underwent septic abdominal surgery decreases caspase-3 protein levels, and therefore, it was presumed to have an anti-apoptotic impact on peripheral blood neutrophils [47].

Neutrophils are considered as the first-line defense of the immune system against infections. They are able to combine and extrude their DNA and bactericidal molecules to produce neutrophil extracellular trap (NET)-like structures in an exceptional type of cell death termed NETosis. This is an important process to control extracellular infections limiting collateral damage. The aberrant function of neutrophils has been implicated in human diseases such as sepsis and autoimmune diseases [87].

Studies have recently argued that vitamin C deficiency in GULO-knockout mice showed enhanced NETosis in the lungs of septic animals and increased circulating cell-free DNA [59]. Some other studies suggested that in vitro supplementation of human neutrophils with vitamin C can attenuate phorbol ester-induced NETosis [74].

HIF-1 is a transcription factor easing neutrophil survival at hypoxic loci by delaying apoptosis. Vitamin C is considered a cofactor for the dioxygenase enzymes containing iron that would adjust the levels and activity of HIF-1 [88]. Under normoxic conditions, vitamin C deficiency in GULO-knockout mice upregulated HIF-1 and attenuated neutrophil apoptosis and clearance by macrophages [89].

Lymphocytes

Lymphocytes are the immune cells that contribute to the generation of antibodies (called B lymphocytes) and cooperate with other immune cells to guide them toward invading pathogens. When they detect a threat, lymphocytes quickly elicit a proliferative response. Vitamin C has been shown to enhance this response, thereby provoking lymphocytic activities [2, 39].

In older adults with disabled lymphocyte proliferation, vitamin C can be used to restore youthful levels of function. Similar enhancements of lymphocyte proliferation have been indicated by administering vitamin C to aging laboratory animals. Diabetes also disables the production of lymphocytes and the function of T lymphocytes. Supplementing diabetic rats with vitamin C increased lymphocyte production from 57% to virtually 100% and led to the generation of non-diabetic immune cells in a living diabetic animal.

Antibodies are considered noncellular components of the immune system. They help the body to detect and kill threats and cancerous cells. Vitamin C treatment can cause an increase in the levels of three main classes of

antibody immunoglobulins: IgA which protects the body against infections mostly on mucosal surfaces, including the respiratory and digestive tracts, IgG which provides long-term protection in the bloodstream, and finally IgM which is the earliest immunoglobulin that appears in blood in response to invading threats. The levels of antibodies and other protective molecules in the blood increased dramatically when human volunteers took 1000 mg doses of vitamin C per day for 75 days. Moreover, several studies have suggested that supplemental vitamin C raises serum levels of antibodies and C1q complement protein in guinea pigs, which—similar to humans—cannot produce vitamin C themselves and are dependent on dietary vitamin C.

NK cells of the immune system move to infectious targets. With aging, NK-cell function declines. Studies show that NK-cell function can be increased with vitamin C. Vitamin C also helps NK cells to chase and kill tumor cells through decreasing the shielding impact of platelets (blood clotting cell fragments) that would avert NK cells from killing them. This effect may be helpful to prevent cancer metastases.

Vitamin C and Inflammatory Mediators

Vitamin C helps to protect immune cells from intracellular ROS produced during an inflammatory response. As a matter of fact, vitamin C may have an important role in the adjustment of the inflammatory response [90]. Some available evidence points that ascorbic acid may have antiviral effects in humans. When incubated with cultured mouse cells in vitro and when administered to mice in vivo, vitamin C can provoke interferon production [91, 92].

T Lymphocytes

There are three main subsets of lymphocytes, i.e., T cells, B cells, and NK cells. The T-cell receptor (TCR) being responsible for detecting and binding specific antigens binds to major histocompatibility complex (MHC) molecules. There are various types of T lymphocytes, including T-helper cells, cytotoxic T cells, and regulatory T cells. T-helper cells adjust immune responses and are CD4-positive cells. Their TCR binds to MHC class II on antigen-presenting cells (APCs). Following the binding, T-helper cells release cytokines that trigger other immune cells, such as Th1 cells, characterized by the secretion of interferon-gamma (IFN-γ); Th2 cells, which release interleukin-4 (IL-4), IL-5, and IL-13; tumor necrosis factor alpha (TNF-α); and cytotoxic T cells. Cytotoxic T cells are characterized by an MHC class I binding CD8 protein on their cell surface. Following the binding, the cytotoxic T cells mature, and by being activated by an infected cell, they release perforin and granzymes that kill the infected cells. Memory T cells are long-living, and they can provide lifelong immunity and also detect previously encountered pathogens. Regulatory T cells end T-cell-mediated immunity toward shutting down an immune response and help tolerate self-antigens [93].

T-Cell Development

T cells stem from hematopoietic stem cells found in the bone marrow. The progenitors of T cells migrate to the thymus. The earliest developing thymocytes do not express coreceptors CD4 and CD8 and are called double negative (DN) [94]. Most cells in the thymus cause αβ T cells to rise. However, about 5% bear the γδ T-cell receptor (TCR). Other cell components of thymus like stromal cells provide a structural support and cytokines considered to be important for the selection of functional T cells [95].

Huijskens et al. discovered that vitamin C can boost human T-cell proliferation in vitro [96]. Most importantly, they pointed out that while culturing T cells from cord blood or G-CSF stimulated hematopoietic stem cells, vitamin C is required in vitro for the transition of DN precursors to the next stage, so-called double-positive (DP, CD4+ CD8+) stage, in feeder-free cultures and also in cultures with stromal cells. They indicated that the initial maturation of T cells after 3 weeks was enhanced under the influence of

vitamin C in a dose-dependent manner with an optimal dose of 95 μM [96]. These findings are consistent with those of mice. In this study, the researchers cultured adult bone marrow-derived hematopoietic progenitor cells on stromal cells and pointed out that these cells are only distinguished from the DP stage when vitamin C is present. Fetal liver chimeric mice were generated by transferring Slc23a2-deficient hematopoietic stem cells (HCS) into recipient mice. Without Slc23a2, hematopoietic cells are not able to concentrate vitamin C. As a result, in animals suffering from a Slc23a2-deficient hematopoietic system, T cells almost did not maturate compared to control mice [95].

Manning et al. [95] suggested that the induction and maintenance of Cd8a gene expression depend on vitamin C-dependent removal of repressive histone modifications, rather than on its function as an antioxidant. This result suggests that in humans and mice, vitamin C is required for the early development of T cells when it overcomes a development block from DN to DP. Additionally, vitamin C accelerates the maturation process of T lymphocytes. This effect is partly due to vitamin C-dependent epigenetic regulation in mice.

T-Cell Proliferation

The literature showed the effect of vitamin C on the proliferation and survival of T cells, both in vitro and in vivo. The number of studies on vitamin C and T-cell proliferation in humans is limited. The elderly people often have lower serum levels of vitamin C and are prone to infections more than other age groups. In a placebo-controlled trial, the elderly received either an intramuscular injection of vitamin C (500 mg per day) or the placebo for 1 month. An increase in T-cell proliferation was observed in the vitamin C-supplemented group in comparison with the placebo group [40].

The study of mice pointed out the impact of vitamin C on activated T cells. More than 70% of apoptotic cells were observed in cultures lacking vitamin C. Additionally, vitamin C treatment (450 μM) reduced apoptosis by one-third [97]. Another study investigating the effects of vitamin C on murine T cells showed that low concentrations (62.5 μM and 125 μM) of this vitamin cannot alter the proliferation or viability of T cells, while higher concentrations (250 μM and 500 μM) could reduce both these parameters [98]. Moreover, researchers in another study found that vitamin C prevents oxidative damage in human T cells. They reported that medium to high concentrations of vitamin C (57–142 μM) decreased T-cell proliferation, and higher concentrations (284 μM) reduced cell viability and IL-2 secretion by more than 90% [99]. Another research investigating the expression of SVCT on T cells showed a similar impact. Peripheral blood T cells from healthy volunteers were activated in vitro in the absence or presence of various doses of vitamin C before and after the activation. Low doses (62.5–250 μM) of vitamin C did not influence proliferation or apoptosis of T cells. The T-cell proliferation was, however, suppressed with high doses (500–1000 μM) of vitamin C. Also, when vitamin C was added before T-cell activation, there was an increase in apoptosis [100].

Another research investigating the effect of vitamin C deficiency on the numbers of lymphocyte in guinea pigs showed that a 4-week vitamin C-deficient diet decreased the number of T lymphocytes, but free-supplemented animals (25 and 250 mg a day) showed a moderate increase in the T-cell numbers [101]. Plasma concentrations of free vitamin C were remarkably lower in animals lacking vitamin C supplement in comparison with vitamin C-treated animals. In another study of vitamin C-deficient SMP30KO$^{-/-}$ mice, the investigators determined the long-term effect of vitamin C on immune cells through a diet with an increased vitamin C level (200 mg/kg vs. 20 mg/kg). Throughout the 1-year study, the number of T lymphocytes in the peripheral blood was increased. Particularly, the numbers of naive T cells, memory T cells in the spleen, and mature T cells in the thymus were increased [23]. Plasma concentrations of vitamin C in mice having a low-dose vitamin C diet were the same as wild-type mice, but plasma concentrations in mice receiving high-dose vitamin C were notably higher.

Badr et al. investigated whether the impaired T-cell function in diabetes type I can be improved by vitamin C supplementation in a streptozotocin-induced diabetes type I rat model. At baseline, animals had lower T-cell cytokine production, lower proliferation, and lower surface expression of CD28, a kind of protein that is necessary for T-cell activation and survival. Vitamin C supplementation (100 mg/kg a day for 2 months) increased cytokine secretion, CD28 expression, and proliferation [102].

T-Helper Cells

Th cells are a kind of T cells that especially contribute to the adaptive immunity. Many researchers indicated that vitamin C induces a shift of immune responses from Th2 to Th1. In these studies, a mouse was used as a model to examine the effect of vitamin C (5 mg a day) on the DTH response against 2,4,-dinitro-I-fluorobenzene (DNFB). Supplementation with vitamin C throughout the sensitization resulted in higher levels of Th1 cytokines (TNF-α and IFN-γ) and lower levels of Th2 cytokine (IL-4) [103]. This vitamin C-induced shift of immune balance from Th2 to Th1 was observed in another study. The effects of vitamin C supplementation on asthma were analyzed. Vitamin C supplementation (130 mg/kg a day for 5 weeks) of ovalbumin-sensitized mice notably increased the IFN-γ/IL-5 secretion ratio in bronchoalveolar lavage (BAL) fluid in comparison with the control group mice [104]. Consistently, several animal studies indicate that vitamin C inhibits Th2 differentiation and an increased Th1 response [17]. Additionally, one study pointed out that Th17 polarization of murine naive CD4+ cells is improved by vitamin C [105]. The authors revealed that this effect may be due to the effects of vitamin C on histone demethylation that would boost the expression of the IL-17 locus.

Regulatory T Cells

Regulatory T cells are a subset of T cells that have an important role in the maintenance of self-tolerance. Therefore, they prevent autoimmune diseases and suppress harmful inflammatory diseases, for instance, asthma and inflammatory bowel disease (IBD).

Vitamin C acts as a cofactor for ten-eleven translocation enzymes [106], a family of proteins that catalyze the first step of DNA demethylation, the alteration of 5-methylcytosine (5 mC) to 5-hydroxy-methylcytosine (5 hmC) [94, 107]. Therefore, vitamin C is an important epigenetic regulator in embryonic stem cells [108]. Addition of vitamin C to embryonic stem cell cultures induced demethylation of more than 2000 genes in an hour [109].

Nikolouli et al. indicated the effect of vitamin C on skin graft rejection in mice after curing with ex vivo cultured alloantigen-induced Tregs. When cultured in the presence of vitamin C, the in vivo alloantigen-induced murine Tregs presented higher DNA demethylation and stability of Foxp3 expression. Moreover, these Tregs had a better suppressive capacity in vivo; therefore, they helped to promote skin allograft acceptance [106, 110]. A serious and sometimes lethal complication following allogeneic hematopoietic stem cell transplantation is called graft-versus-host disease (GVHD) which is caused by all reactive donor T cells inducing tissue injury in the recipient. In this model, when Tregs transferred into mice with acute GVHD, in vitro murine alloantigen-induced Tregs pretreated with vitamin C caused more stable Foxp3 expression and could effectively lessen GVHD symptoms. Furthermore, cultured human alloantigen-induced Tregs had a higher Foxp3 expression if they were cultured with vitamin C. Vitamin C is able to regulate Tregs function through epigenetic regulation of the master transcription factor Foxp3. In this manner, vitamin C would benefit the generation of ex vivo alloantigen-induced Tregs that can be applied for clinical purposes in transplantation and autoimmune disorders [17].

B Lymphocytes

B lymphocytes exist in the blood, lymph nodes, spleen, tonsil, and other mucosal tissues. They constitute approximately 5–25% of human blood lymphocytes. B cells originate in the bone marrow in humans, developing from a common progenitor shared with NK, T, and some DC subsets. B lymphocytes can differentiate into antibody-secreting

plasma cells that help the immune system to protect the body against invading pathogens.

A 4-week vitamin C-free diet showed a continuous increase in the percentage of B lymphocytes whenever decreasing the percentage of T lymphocytes [101]. Mouse spleen B cells were cultured with an anti-μ antibody in the presence of stimulating cytokines for 2 days, and afterward, they were washed and recultured with and without the stabilized form of vitamin C, AA-2G (ascorbic acid-2-glucoside vitamin C). In the absence of vitamin C, the number of viable cells was reduced much faster. AA-2G also increased the generation of IgM in a dose-dependent manner [111]. Another research investigated the effect of vitamin C on mouse spleen B cells in vitro. Pre-treatment with vitamin C resulted in a small dose-dependent increase in apoptosis (16% at a concentration of 1 mM) of murine IgM/CD40-activated B cells [112].

Amakye et al. suggested that the effect of vitamin C supplementation might be useful for vaccination against infectious bursal disease. Vitamin C supplementation (1 g/mL) led to an increase in immunoglobulin levels [113]. Interestingly, chickens that were not vaccinated but received vitamin C supplementation did not present any symptoms, and none of them were dead after the challenge with infectious bursal disease. However, all non-vaccinated chickens which did not receive vitamin C supplementation experienced clinical symptoms, and 70% of them survived after the challenge with infectious bursal disease [113]. Studies in guinea pigs indicated that after immunization with sheep red blood cells and bovine serum albumin [114, 115], high dose of vitamin C supplementation increased immunoglobulin levels. Albers et al. pointed out the impact of high-dose vitamin C (2500 mg a day for a month) in mice sensitized by topical usage of dinitrochlorobenzene (DNCB) and rechallenged 2 weeks later. The researchers suggested that high-dose vitamin C supplementation had no significant effect on immunoglobulin production [116].

Vallance et al. found a correlation between plasma vitamin C concentrations and serum immunoglobulin (IgG and IgM) levels. Daily supplementation with 1 g ascorbic acid for a week significantly boosted serum IgG as well as plasma and leukocyte vitamin C concentration in healthy people [117]. Another study in healthy volunteers indicated an increase in serum levels of IgM and IgA after daily supplementation with 1 g ascorbic acid for 75 days [118]. However, these results were not replicated by Kennes et al. [40]. In a placebo-controlled trial in the elderly, participants received either an intramuscular injection of vitamin C (500 mg a day) or a placebo for 1 month. Vitamin C showed no effect on serum IgA, IgM, and IgG levels.

Natural Killer Cells

NK cells are considered as lymphocytes. They are in the same family as T and B cells, and they come from a common progenitor. However, NK cells are categorized as group I innate lymphocytes (ILCs) and respond rapidly to a wide range of pathological challenges [119]. Huijskens et al. pointed out that ascorbic acid (95 μM) can raise the proliferation of NK cells. They showed that ascorbic acid increases the production and expansion of NK-cell progenitors from hematopoietic stem cells and from T-/NK-cell progenitors in vitro in a cytokine-stimulated culture [120].

Farmakis et al. analyzed and isolated NK cells from peripheral blood of patients suffering from β-thalassemia major. The cytotoxic function of NK cells is profoundly impaired in patients suffering from β-thalassemia compared with healthy subjects. It is at least in part due to oxidative stress caused by iron overload after multiple blood transfusions [121]. Treatment with ascorbic acid (200 μg/mL) could restore the cytotoxic capacity of NK cells in patients with β-thalassemia, while the NK-cell function of healthy controls remained unchanged [122]. In this case, the positive effect of ascorbic acid on the NK-cell function is probably associated with its antioxidant properties. NK cells in patients with iron overload are known to produce more intracellular ROS [123].

Vojdani et al. analyzed the effect of vitamin C on the NK-cell function. NK cells from healthy

humans supplemented with a single high dose of vitamin C showed a biphasic effect of vitamin C on NK-cell cytotoxicity: a small decrease 1–2 hours after supplementation and a significant increase after 8 hours, with a maximum effect after 24 hours, and return to baseline function after 48 hours [93].

Another study investigated the effect of buffered vitamin C on NK-cell, T-cell, and B-cell functions in patients exposed to toxic chemicals. NK-cell function was dramatically reduced after being exposed to different chemical toxins. When the blood was drawn for the first time, 55 patients drank granulated buffered vitamin C in water quickly at an intake dosage of 60 mg/Kg body weight. After 24 hours, blood was again drawn for a follow-up study of NK-cell, T-cell, and B-cell functions. A high oral dose of vitamin C was able to boost NK-cell activity up to tenfold in 78% of patients. Lymphocyte blastogenic responses to T- and B-cell mitogen were restored to the normal level after vitamin C application [124].

Siegel et al. analyzed the effect of including vitamin C in the drinking water on NK-cell activity in three highly inbred strains of mice. Vitamin C treatment did not have any effects on the functions of natural killer cells in the tumor- and autoimmune-prone NZB strain and also in the normal and low tumor-incidence DBA/2 and BALB/c mice [125].

Vitamin C and Immune-Related Disorders

Common Cold

One of the most widely known health beneficial effects of vitamin C is the prevention and treatment of the common cold [27, 126]. Studies indicate that vitamin C can ameliorate symptoms and reduce the duration of the common cold [127]. However, less is understood about whether supplementation with vitamin C can also reduce the incidence rate of colds [27, 128]. Randomized and non-randomized studies using 1.0 g/day or more have shown that vitamin C can reduce the severity and duration of the common cold, but had no significant effect on the incidence of common cold [27, 80]. However, there are studies using higher doses of vitamin C that showed a reduction in the incidence of colds [126, 127]. Additionally, some studies have provided evidence that vitamin C supplementation can reduce cold incidence among people with heavy stress, such as soldiers, athletes, and individuals with chronic stress or obesity. Cold symptoms and duration were also remarkably reduced in the supplemented versus the placebo subjects [129, 130].

In recent years there has been a considerable debate over the role of vitamin C in enhancing immunity during the common cold [36]. Sufficient dose of vitamin C is believed to provoke the immune response by enhancing T-lymphocyte proliferation (by affecting the cell survival and apoptosis signaling) and influencing T-lymphocyte function in response to infection [97, 131]. T lymphocytes are known to mediate the direct lysis of infected cells by producing a number of important cytotoxic proteins or activating B lymphocytes to produce antibodies to control the infection [132].

Studies demonstrate that older adults display lower amounts of vitamin C in their blood circulation and immune cells. This may explain why cold infections can trigger the onset of severe bacterial infections such as pneumonia or bronchitis in the elderly people [131, 133, 134].

Infection

Various factors determine whether an individual will get sick or not. However, the immune system remains the body's first defense against invading pathogens [2]. Accordingly, it is of utmost importance to sufficiently nourish the immune system with micronutrients required to promote appropriate functioning. Vitamin C is commonly consumed by most of the major cell types of the immune system involved in controlling infections [2]. In this manner, during episodes of acute severe infection, the body store of vitamin C may

be depleted, probably via exacerbated inflammation and metabolic needs [35, 131].

Numerous studies have investigated the effects of vitamin C on infections. As reviewed by Hemilä (2017), there have been more than 140 animal studies indicating the effect of vitamin C in alleviating symptoms, shortening duration, and preventing infections caused by virus, bacteria, and protozoa [2, 131].

However, human studies are conflicting. Three controlled trials have shown that vitamin C supplementation is effective in ameliorating symptoms of upper respiratory tract infections and can decrease pneumonia incidence by up to 80% [131]. This is a promising result for older individuals since the mortality rate from severe pneumonia among elderly people exceeds 16% (even with antibiotic treatment). In the respiratory tract, pulmonary macrophages require vitamin C to orchestrate protection against airborne viruses and bacteria, especially when the invading pathogen begins to spread throughout the body [35].

Herpes zoster (HZ) results from reactivation of varicella-zoster virus (VZV). Acute infection with VZV often leads to long-lasting postherpetic neuralgia (PHN). A recent study revealed that patients with PHN had notably lower plasma levels of vitamin C than healthy individuals. Controlled trials also showed that vitamin C administration considerably alleviated pain related to PHN and HZ [131, 135].

As discussed above, during episodes of acute severe infection, the body stores of vitamin C may be depleted, probably by exacerbating inflammation and increasing metabolic demands. Supporting this, concentrations of vitamin C in plasma and leukocytes would drop rapidly with the onset of infection and be restored to normal levels with the amelioration of symptoms proposing that vitamin C plays a crucial role in the recovery process [2]. Prophylactic trials on prevention of infection suggested that adequate dietary intake of vitamin C maintains normal levels of vitamin C at cellular and tissue levels. However higher doses of vitamin C are required to treat infections by modulating inflammatory response and therefore decreasing metabolic requirements [35]. In contrast, a vitamin C-deficient diet would increase susceptibility to infections. Current evidence suggests that vitamin C benefits individuals with weakened immune function. For example, in patients with recurrent skin infections who had impairment in neutrophil recruitment and killing of microorganisms, vitamin C with lower side effects was as efficient as a potent immune-regulating drug, levamisole, at improving neutrophil activity and induction of long-lasting remission. In patients with recurrent furunculosis, similar improvements in neutrophil function and clinical status were seen by administration of 1 g/day vitamin C supplement [35, 73].

Cancer

Cancer refers to a set of diseases characterized by loss of cell growth control with the ability to invade surrounding normal tissue or spread to distant parts of the body [136, 137]. The substrates, intermediates, and products of cellular metabolism have the potential to influence cellular identity. Therefore alterations in cell metabolism may have a crucial role in tumor progression and transformation to cancer [24, 138].

In 1949, vitamin C was first proposed to have a preventive role against cancer and has been the subject of extensive research in this regard. Although it was at first thought that vitamin C might combat cancer by supporting collagen synthesis and thereby preventing tissue invasion and metastasis, it has become increasingly clear that several mechanisms can account for the involvement of vitamin C in the treatment and prevention of cancer [27, 36, 139]. These mechanisms include antioxidant effects, inhibition of formation of nitrosamines, enhancement of the immune response, and epigenetic regulation [140–142]. One of the most important anticancer properties of vitamin C is neutralizing free radicals before they can damage DNA and initiate tumor growth [27].

Recent experimental studies have revealed that a high concentration of vitamin C can lower or even eliminate the growth of various types of cancers [36]. There is evidence linking high intake of vitamin C with a reduced risk of cancers

of the esophagus, oral cavity, pancreas, cervix, stomach, and breast and also non-hormonal cancers [36]. Despite selective cancer cell toxicity induced by high-dose vitamin C treatment in vitro and in mouse models, the molecular mechanisms underlying the anticarcinogenic potential of ascorbic acid in different types of cancer cells are not completely elucidated [143]. However, evidence indicates that vitamin C can act as a scavenging factor for ROS and inhibit the formation of potentially carcinogenic N-nitroso compounds from nitrates and nitrite in the stomach. Consequently, vitamin C may help to protect against stomach cancer [27].

Recently, Yun et al. demonstrated that high-dose vitamin C treatment can induce cell death and encourage apoptosis by induction of oxidative stress and inhibition of glycolysis in glycolysis-addicted KRAS- and BRAF-driven cancer cells [143].

Recent research has found that immature stem cell population and progenitor cells contain higher levels of vitamin C (about 2 and 20 times) compared with differentiated cells. It appears that high levels of vitamin C are required to regulate the number and differentiation of hematopoietic stem cells, mainly via influencing the Tet2 protein. Tet2 is a key regulator of the gene expression by affecting methyl groups and thereby modulating DNA modification. Vitamin C is thought to be a cofactor for the enzymatic activity of Tet2. It is thus expected that vitamin C deficiency may enhance the self-renewal potential of HSC conferring an increased susceptibility to develop leukemia [24].

Immunonutrition plays a crucial role in cancer therapy [7]. Deficiency of vitamin C is relatively rare in the general population but commonly occurs in patients with cancer [142]. Vitamin C might be useful for the treatment of advanced cancer.

High-dose vitamin C administration has been investigated as a treatment for patients with cancer since the 1970s. High-dose vitamin C as a supportive therapy may be given orally or intravenously (IV) before and after chemotherapy [142]. Studies have shown that IV injection is about 70-fold more effective to increase serum levels of vitamin C compared with oral administration [36, 144]. IV administration of vitamin C is a safe supportive approach to reduce inflammation in patients with cancer and helps to ameliorate symptoms linked to antioxidant deficiency, disease processes, and side effects of standard cancer treatments [144]. In contrast to levels within the normal physiological range (0.1 mmol/L), pharmacological amounts of vitamin C (0.3–20 mmol/L) can selectively destroy tumor cells in vitro. Thus, it seems that high levels of vitamin C induce the formation of hydrogen peroxide and provide its implication as a prodrug concept in cancer therapy [36].

In addition to the antioxidant effect of vitamin C, this micronutrient is a vital factor for the functional network of epigenetic regulators [142]. Vitamin C is a cofactor for Fe^{2+}- and α-ketoglutarate-dependent dioxygenases which include an enormous number of various enzymes, such as collagen prolyl hydroxylases and epigenetic regulators [142, 145]. Epigenetic dysregulation represents a driving mechanism of malignancy, and vitamin C could be considered as an epigenetic anticancer agent [145]. Recent studies have shown that vitamin C at levels within the physiological range combined with hypomethylation factors may have a synergistic function to induce DNA demethylation. In recent years researchers have begun to focus on the potential role of vitamin C to optimize the outcomes of epigenetic therapy in patients with cancer and alternatively to act as a high-dose nutrient therapy [142].

Vitamin C is able to cooperate with the immune system in attacking cancerous cells. Kim et al. investigated whether vitamin C depletion is responsible for the inhibition of NK-cell-mediated antitumor activity in a mouse model of ovarian cancer. Compared to NK cells from vitamin C-supplemented GULO−/− mice and wild-type mice, NK cells isolated from GULO−/− mice exhibited a significant decrease in killing capacity, reduced expression of the activating receptors CD69 and NKG2D, and reduced production of the cytolytic proteins perforin and granzyme B [146]. Also, vitamin C can enhance the antitumor

activity of NK cells by debilitating the shielding property of blood platelets that would avert NK cells from destroying cancerous cells [147, 148].

As discussed, there are a variety of mechanisms proposed to underlie the role of vitamin C in cancer. Additionally, vitamin C in the form of dehydroascorbic acid (DHA) can directly import into the cancerous cells. Altered (reduced or enriched) intracellular vitamin C within tumor cells may affect the clinical outcome of chemotherapy or radiation therapy. As a consequence, vitamin C administration may reduce the effectiveness of anticancer therapy, because this micronutrient acting as a potent antioxidant can neutralize oxidative stress induced by chemotherapy. Further studies are needed to characterize the kinetics of the effect of vitamin C in cancer [27].

Allergy, Inflammation, and Autoimmunity

Impairment in antioxidant capacity can cause hypersensitivity, inflammatory, and "autoimmune" conditions [149]. Vitamin C is a potent antioxidant. High-dose vitamin C can help with the treatment of nasal allergic symptoms [150].

Mast cells are tissue-resident immune cells that upon stimulation secrete mediators involved in allergy and inflammation [151]. Vitamin C prevents lipid peroxidation of membrane phospholipids and shows free radical scavenging activity. Vitamin C depletion has been associated with elevated blood levels of histamine (histaminemia) which has been reported to damage endothelial-dependent vasodilation. However, the effect of vitamin C on allergic and inflammatory diseases has not been fully understood yet [152, 153].

Studies suggest that the transcription factor NF-κB signaling cascade promotes inflammation [154]. High-dose vitamin C by inhibition of NF-κB signaling can cause a modulatory effect on the immune system. The exact mechanism of action of vitamin C remains the subject of current investigation, but p38 MAPK is proposed as a potential intracellular target of vitamin C [155].

A malfunctioning immune system can lead to autoimmune diseases where the immune system produces autoreactive reactions that attack host cells, tissues, and organs [156]. Among the first studies of the effect of vitamin C in autoimmune diseases is the study performed by Animashaun and his colleagues in 1990 on IBD. The study reported that vitamin C can exert immunoregulatory effects on T-cell function in Crohn's disease [157].

The potential role of vitamin C in the nervous system diseases has been investigated for many years [158, 159]. The central mechanisms that have been implicated in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis (MS), and v lateral sclerosis, include altered redox homeostasis and abnormal accumulation of proteins [160]. Of note, vitamin C is known to act as both a neuromodulator and a scavenger for ROS [160, 161]. In this manner, vitamin C can help to diminish neurodegenerative processes and ameliorate the progression of neurodegenerative diseases [160].

MS is an autoimmune disease where the immune system attacks the myelin sheath covering the axons in the brain and spinal cord [162, 163]. In demyelinating diseases, such as MS, myelin sheaths formed by oligodendrocytes play a vital role in neural functions through the restoration of myelin sheaths [164–166]. Remyelination needs differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes (OL) [167]. A new study using a high-throughput screening system reported that L-ascorbyl-2-phosphate (As-2P), a stable form of vitamin C, is largely involved in the induction of differentiation of OPC in OL. As-2P has been shown to facilitate the formation of myelin sheaths and promotes the repair of the myelin sheaths. Further work needs to be performed to investigate whether vitamin C can be used as an alternative medicine for treating demyelinating diseases [168].

Systemic lupus erythematosus (SLE) is another autoimmune disease, and its pathogenesis mainly involves oxidative stress [169–171]. A recent study has shown that long-term treatment

with antioxidant vitamins including vitamin C can reduce oxidative stress and lipid peroxidation in patients with SLE [171].

Aging

Immune functions decline with age, which is referred to as "immunosenescence" [172]. Major age-related changes involving the immune system occur in the mass of thymus gland, the proportion of T-cell subsets, DTH response, T-cell proliferative response, and to a lesser extent in B-cell function and innate immune functions [172]. In contrast, the number and proportion of late-stage memory T cells and B cells commonly increase with age, being particularly prominent in the CD8+ cytotoxic T-cell pool [173].

Compared with younger adults, elderly people experience significantly lower levels of vitamin C circulating in their serum and immune cells [134, 174]. Malnutrition commonly occurs in the elderly and can worsen the severity of age-associated immune deviation [173]. The interactions between aging, nutrition, and immunity have been studied extensively over the past few decades. The conclusion from the majority of these studies is that both aging and vitamin C deficiency can result in immune dysfunction involving the composition, function, and competence of the human immune system [40, 173]. In aging people, vitamin C deficiency broadly affects the different key features of the immune system. White blood cells from older adults often perform poorly in vivo in response to stimulation by antigens (foreign substances). Furthermore, in vitro proliferative activity of lymphocytes to mitogenic stimulation by concanavalin A (Con A) is greatly reduced in the elderly compared to young individuals [175]. From a biological perspective, these age-associated changes in the immune system leave older individuals vulnerable to infectious and noninfectious diseases [173].

Vitamin C can be used to boost immune function in the elderly. When lymphocytes from older adults were preincubated overnight and cultured in a solution enriched with vitamin C (10 μg/ml), the function and proliferation of these cells were restored to that of cells from youthful people. In a placebo-controlled study, vitamin C enhanced the proliferative response of T lymphocytes in vitro as well as the hypersensitivity response to the tuberculin test in vivo. Neither serum concentrations of immunoglobulins (IgA, IgG, and IgM) nor the proportion of E-rosette-forming cells were modified [40]. Studies in individuals who were an average of 72 years old indicated that peripheral immune response (the phagocytic activity of blood WBC and splenic PMN) was restored after vitamin C administration [176]. Aged animals supplemented with vitamin C also showed enhancements of lymphocyte proliferation.

Conclusions

Vitamin C is important to many functions in human biology. In addition to its important role in a number of metabolic functions and protecting the body against oxidative challenges, vitamin C has immune-enhancing/immune-modulating properties in both innate and adaptive immunity. It has conclusively been shown that that vitamin C is necessary to mount and maintain proper immune response against pathogens, and inadequate intake or plasma levels of vitamin C lead to a higher risk of infections especially in the upper respiratory tract such as the common cold and influenza. According to the experimental evidence, micronutrient supplementation with vitamin C, especially in groups such as the elderly, has been shown to reduce the duration and severity of cold symptoms by enhancing various immune cell functions. Ensuring sufficient vitamin C levels may be particularly an essential factor in conditions involving additional challenges in the immune system such as inflammation, autoimmunity, and cancer. Since the molecular mechanism underlying cancer-fighting potential of vitamin C is not clearly understood, the link between vitamin C and cancer, as a very challenging disease in human, is still under investigation. More mechanistic in vitro and in vivo studies are required in supporting the beneficial claims on vitamin C.

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Vitamin B12, Folic Acid, and the Immune System

6

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Contents

Key Points

- Folic acid (vitamin B9) and cobalamin (vitamin B12) play a crucial role in the healthy balance of the immune system.
- Inadequate levels of folic acid and B12 can drastically alter immune response.
- Hyperhomocysteinemia that occurs due to deficiency of folic acid and B12 causes systemic and vascular inflammation contributing to the pathogenesis of many other diseases such as cardiovascular, kidney, and neurovascular diseases, osteoporosis, and cancer.
- Adequate dietary levels of folic acid and B12 can act as preventative measures for inflammation, immune dysfunction, and disease progression.

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Introduction

General Functions of B9 and Effects of B9 Deficiency

Vitamin B9 (folate, folic acid) has an array of important functions within the body. In its primary coenzyme form, tetrahydrofolate (THF) aids in metabolism by facilitating a series of one-carbon transfer reactions. During methylation reactions, it works with B12 and B6 to recycle homocysteine (HCY) into methionine. Folic acid is a vital component for cellular function and division and assists DNA synthesis, repair, and methylation. In the form of 5-methyl tetrahydrofolate (5-MTHF), folate can assist in regulation of monoamine neurotransmission and breakdown of and synthesis of certain neurotransmitters including norepinephrine, dopamine, and serotonin. Folic acid and B12 work synergistically by aiding the conversion of each other to a coenzyme form. Folic acid found in food as poly-glutamates needs to be broken down by the body into mono-glutamates prior to absorption taking place in the intestines. Folic acid is excreted from the body via the urinary tract [1–4].

Folic acid is found in a variety of food sources including fortified grains, leafy green vegetables, legumes, seeds, and liver. Symptoms of folate deficiency include shortness of breath, mental confusion, irritability, depression, weakness, fatigue, headache, and elevated HCY levels. Folic acid deficiency can lead to a variety of disorders including homocysteinemia, anemia, cognitive defects, cardiovascular disease, and cancer and has even been linked to dementia and Alzheimer's disease (AD) [3, 5, 6]. In pregnancy, folate deficiency can result in neural tube defects in the fetus [7]. The clinical form of folic acid deficiency is megaloblastic anemia, and patients who suffer from this condition have demonstrated depressed immune responses which are reversible with folic acid supplementation [8].

General Functions of B12 and Effects of B12 Deficiency

B12 is a vital nutrient required by the body for many important processes. B12 aids in the function of the nervous system by providing nerve cell maintenance and cooperating with cell synthesis as well as the catabolism of fatty acids and proteins. The metabolically active form of B12 is cyanocobalamin. Methylcobalamin, cob(I)alamin, 5′-deoxyadenosylcobalamin, and hydroxycobalamin are other forms of B12 which are found naturally in biological systems. B12-related compounds have a cobalt-centered corrin nucleus with reactive C-Co bonds which participate in isomerase and methyltransferase reactions. These reactions take place in the extraction of energy from proteins and fats. An intimate relationship exists between B12 and folic acid as each depends upon each other for activation. B12 is required to be obtained through dietary sources as it is not synthesized within the body.

The main source of B12 in the human diet comes from animal origin, and the presence of B12 in animal flesh is mostly dependent on the process of biomagnification through food chains [9]. Vegan and vegetarian sources of B12 can be found in nutritional yeast and some yeast-based spreads which have been fortified with B12. Interestingly vitamin B12 is synthesized in large amounts by bacteria in the human colon but this is not absorbed by the body. A study on vitamin B12-deficient vegan patients in the 1950s demonstrated that vitamin B12 deficiency could be cured by feeding these patients watery, B12-rich extracts of their own stools [10]. It is common to find vitamin B12 deficiency, particularly in the metabolically related B vitamins B12, B6, and B9, in the elderly. This is primarily due to malabsorption, poor intake, or higher requirements.

Vitamin B12 absorption within the body relies on intrinsic factor released by parietal cells within the stomach. The B12 content within food is bound to proteins which are released by hydrochloric acid and proteases. After release from food, intrinsic factor forms a complex with B12 which allows it to be absorbed within the ileum. The amount of absorption is reliant on the capacity of the intrinsic factor. Once absorbed by the ileum, plasma transporter, transcobalamin II, transfers the B12 into cells where it is degraded by lysosomal activity and the free B12 travels into the cytoplasm. Excretion of B12 occurs mostly in bile whereby it is reabsorbed and stored

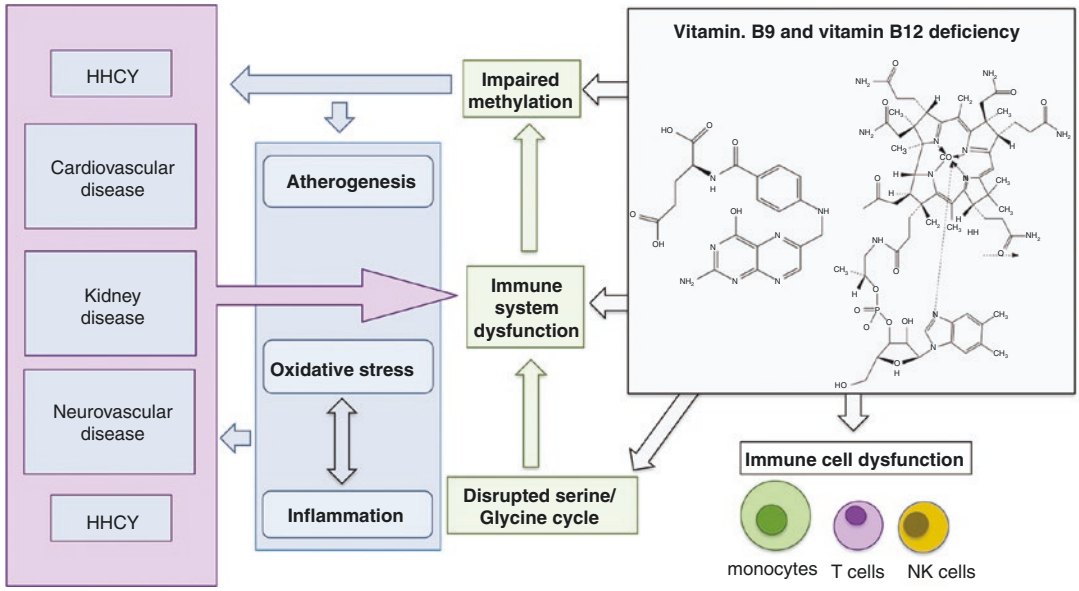

Fig. 6.1 Effects of folic acid and vitamin B12 deficiency on immune homeostasis

by the liver. B12 taken in excessive amounts is excreted in urine [2, 4, 9–11].

Vitamin B12 deficiency symptoms include peripheral tingling, soreness of mouth and tongue, weakness, fatigue, loss of appetite, weight loss, and constipation. B12 is responsible for the production of high-turnover cells such as red blood cells. Therefore, in deficiency states, diseases such as megaloblastic or pernicious anemia can ensue. B12 deficiency affects DNA production by causing folate to be trapped in its inactive form. Within the immune system, B12 deficiency is correlated with a disturbance in immune cell function including a reduction in $CD8^+$ and natural killer (NK) cells and increased synthesis of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 by macrophages [12].

Metabolic Impacts of B9/B12 Deficiencies on Immune Health

Serine/Glycine Cycles

One metabolic impact of folic acid and B12 deficiency which can compromise immunity occurs within the serine/glycine cycle. Serine is a nonessential amino acid derived from dietary glucose. Certain tissues within the body produce glycine while others produce serine from glycine. Both serine and glycine are transported rapidly across the mitochondrial membrane. Folic acid plays an important intermediatory step in the conversion of glycine to serine and folic acid deficiency renders glycine incapable of producing serine. This can result in the inadequate implementation of many metabolic processes including impaired RNA and DNA function, fat and fatty acid metabolism, and muscle formation. Serine is needed in the production of tryptophan which in turn is needed to produce serotonin. Decreased serotonin or tryptophan can lead to depression and other mood disorders [13–17]. Low serine can also interfere with the immune system function by preventing proper antibody formation and interfering with the proper functioning of effector T cells (Fig. 6.1)[18, 19].

Methylation

Methylation is a vitally important metabolic process which occurs within every cell and tissue of the body. It involves the transfer of a methyl group (one carbon and three hydrogens) into amino acids, proteins, enzymes, or DNA. Methylation

reactions are involved in most chemical reactions occurring in the body and help to regulate healing, energy production, genetic expression, neurological function, and liver detoxification and play a major role in immunity.

The process of methylation takes place within two cycles of events: the SAM cycle (S-adenosylmethionine) and the folate cycle. The enzyme methionine synthase is responsible for catalyzing the methylation of HCY to methionine. Methionine is required to run SAM and the synthesis of methionine requires vitamin B12. The folate cycle requires vitamins B9, B6, and B12 as well as methyl tetrahydrofolate (meth-THF) and NADPH.

Folic acid and B12 deficiencies can cause ineffective methylation reactions leading to a rise in HCY levels in the body which in turn can result in many health disorders including immune dysfunction. As the folate cycle is critical for the maintenance of numerous cellular pathways including DNA manufacture and repair, protein production, cell growth and division, and methylation, it follows that folate deficiency within the diet can adversely affect the proper functioning of these pathways. If folate is unavailable to these processes, metabolic reactions are slowed down resulting in an accumulation of reaction by-products which can disrupt homeostasis and lead to immune dysfunction. Immune dysfunction can present as improper antigen presentation, disturbed cytokine production, unmodulated autoimmune responses, disruptions in immune cell function, and ineffective viral clearance [20]. As the relationship between folic acid and B12 is an essential component of methylation reactions, B12 deficiency will also contribute to disorders in methylation and immune function (Fig. 6.1).

Hyperhomocysteinemia (HHCY)

HCYis a degradation product of methylation. Folic acid and B12 work synergistically to keep HCY levels in check and are the primary nutritional determinants of HCY levels within the body. The negative effects of high HCY are well documented, and hyperhomocysteinemia (HHCY) can result in systemic and vascular inflammation which leads to many health disorders including neurological diseases, osteoporosis, cardiovascular diseases, renal diseases, inflammatory bowel disease, and cancer [21].

HCY is a potent excitatory neurotransmitter. It binds to the N-methyl-D-aspartate (NMDA) receptor when excess HCY levels accumulate in the blood. This binding can lead to oxidative stress, cytoplasmic calcium influx, cellular apoptosis, and endothelial dysfunction. An influx of calcium into the cytoplasm of the cell can react with cytoplasmic phosphate leading to calcium apatite deposition which in turns contributes to depletion of cellular ATP. Oxidative stress then occurs due to the failure of ATP synthesis and accumulation within the cell of reactive oxygen radicals. Oxidative stress can also provoke certain mechanisms which inhibit cellular respiration and exert carcinogenic and atherogenic effects (Fig. 6.1).

Atherogenesis is part of an acute inflammatory response involving monocyte and lymphocyte adhesion to endothelial cell surfaces, monocyte migration into sub-endothelial spaces, macrophage differentiation, and the formation of "foam cells" that, together with T lymphocytes, form fatty streaks and atherosclerotic plaques which are the primary cause of cardiovascular disease [22, 23].

Oxidative stress which follows as a result of increased HCY levels is thought to occur as a result of auto-oxidation of thiol groups in extracellular fluid and plasma. This auto-oxidation mechanism causes the generation of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and hydroxyl radicals [24]. Several studies have shown this to occur in cultured smooth muscle cells [25] and aortic endothelial cells [26]. This increased oxidative stress alters the availability and action of nitric oxide and an influx of leukocyte-recruiting, adhesion molecules which can result in endothelial dysfunction [27]. Within the cell cytoplasm, oxidative stress is more likely to occur from calcium influx as a result of excitotoxic activation of NMDA receptors by HCY [22]. Calcium hydroxyl apatite, an insoluble salt, is formed which inhibits oxidative phosphorylation, thus depleting phosphate levels and causing failure of

ATP synthesis and the accumulation of oxygen radicals and oxidative stress. Immune dysfunction arising from oxidative stress can cause impairment in production and function of key immune cells and an imbalance in inflammatory mediators.

Cardiovascular Disease (CVD)

In 2012, WHO (World Health Organization) pronounced CVD as the most common underlying cause of death worldwide. Several meta-analyses have demonstrated that increased blood HCY levels are the most important risk factor for CVD, stroke [28–30], and ischemic heart disease (Fig. 6.1) [31]. HCY causes damage to cells and tissues of arteries by stimulating the release of inflammatory mediators such as cytokines and cyclins which can result in endothelial cell damage and a reduction in vessel flexibility [32]. HHCY can promote inflammation and enhance the adverse effects on cardiovascular health, caused by smoking and hypertension [33, 34].

A Nobel Prize was awarded in 1995 to two physicians, Brown and Goldstein, who correlated arterial disease with high circulating levels of cholesterol in the blood [35]. The low-density-lipoprotein (LDL) receptor hypothesis states that atherosclerosis occurs as a result of high concentration of blood LDL cholesterol. This hypothesis also states that the lowering of blood LDL cholesterol can bring about a reversal of this pathology. It has since been acknowledged that Brown and Goldstein's theory was wrong and unfortunately generated much fear in the general public about the consumption of cholesterol which still lasts to this day [36]. Few studies have shown an association between elevated LDL levels in the blood and a degree of endothelial dysfunction and hypercholesterolemia is not a risk factor for cardiovascular disease [37–40]. Furthermore, cholesterol consumption has failed to produce vascular accidents in laboratory animals, and the avoidance of cholesterol-containing foods can lead to malnutrition due to under-consumption of fat-soluble vitamins [36].

It has been known for many years that atherosclerotic plaques contain inflammatory cells. As far back as 150 years ago, Virchow speculated that endothelial damage leads to an increased permeability of arterial intima with increased infiltration of plasma and plasma fats associated with degeneration of the arterial wall [41]. Current knowledge of HHCY disease pathology supports this speculation. It is now understood that high blood HCY along with infection and inflammation can be a contributing factor to endothelial dysfunction and atherogenesis [22]. In addition to cardiovascular disease, HHCY has been implicated in other diseases of vasculopathic or microangiopathic origins, such as kidney or neurovascular diseases.

Kidney Disease

Oxidative stress (inducement of endothelial dysfunction) is also a factor of the pathology of chronic kidney disease (CKD). Certain conditions associated with causing oxidative stress, including HHCY, contribute to inflammation and accelerate renal injury progression in CKD [42]. Eighty-five percent of patients with CHD present with HHCY. Reduced kidney function is associated with an increase in CVD, and the association is such that more people are likely to die from CVD than progression to end-stage renal disease. It has been shown that classical and intermediate macrophages are all induced in CVD and further elevated in CKD, due to the systemic/vascular inflammation and increased cytokine production due to HHCY [43]. Interestingly however, another study found that vitamin B6 status played a significant role in relation to inflammatory response in CKD independent of HCY levels [44]. There is indeed a lack of understanding of the complete mechanistic pathophysiology of HHCY, and it appears that the strong protective influence of folic acid and B12 against HHCY is also linked to other anti-inflammatory mechanisms.

Neurovascular Disease

There are much epidemiological evidence and longitudinal data which attribute to elevated

HCY levels as a probable risk factor for cognitive impairment and AD as well as other neurodegenerative disorders such as Parkinson's disease, depression, and dementia [16, 45]. In fact, brain atrophy, oxidative stress, DNA damage, increased apoptosis, excitotoxicity, and neurodegeneration can all occur as a result of elevated HCY [45–48], and the toxic effect to vascular endothelial and neuronal cells is well documented [49, 50]. Ravaglia et al. noted that the risk of AD doubles in patients with elevated HCY [51]. Another study has documented dementia and AD patients as having significantly higher HCY while serum folate and B12 levels are significantly lower [52]. In the elderly population, the status of B vitamins is often found to be inadequate due to a decrease in absorption or a decrease in intake, and there is a correlation between low folate levels and cognitive decline. Folate supplementation in the elderly may be of prime importance in preventing common forms of dementia. In a study of 166 dementia and AD diagnosed patients, folate levels were significantly and consistently higher in the control group than in patients already suffering from a cognitive decline [53]. Another study showed lower rates of brain atrophy in individuals over 70 years old with mild cognitive impairment who were taking supplements of folic acid, pyridoxine, and vitamin B12 [1]. Weekman et al. reported that HHCY can induce pro-inflammatory changes within microglia and astrocytes, relevant to their interaction with vasculature, which would highlight how neuroinflammation caused by HHCY can adversely affect neurovascular disorders[54].

Osteoporosis

Enhanced osteoclast activity, bone reabsorption, and inhibition of bone formation have been demonstrated in some people with even mildly increased concentrations of HCY [55, 56]. Increased HCY levels have also been shown to interfere with the activity of lysyl oxidase, an enzyme required for collagen cross-linking which helps to form the bone matrix [57, 58]. Furthermore, an increase in intracellular calcium due to increased HCY can lead to intramitochondrial stress and HCY-induced bone remodeling via mitochondrial pathways [59]. It is not clear whether folic acid and B12 deficiencies, however, are directly linked to fracture risk or the actual role each of these vitamins plays on the bone or whether the effects of the vitamins occur only via HCY concentrations [60].

Cancer

There is a link between folic acid and B12 deficiencies, increased HCY levels, and cancer. In newly diagnosed lung cancer patients, plasma folate levels were significantly lower and mean HCY levels were significantly higher compared to age-matched healthy control group. There was no difference between the groups in regard to B12 levels. These results indicate that high HCY and low folate could potentially be associated with lung cancer, although further studies are required to consolidate these findings [61]. Likewise, in breast cancer cell lines MCF-7 and MDA-MB-231, epigenetic modulations of RASS-F_1 and $BRACA_1$ occurred as a result of elevated HCY levels, and elevated plasma HCY levels were found to be correlated with increased risk of colorectal cancer in women [62]. HCY levels have also been shown to play a role in estrogen-induced hormonal cancers. Elevated HCY levels can inhibit the O-methylation of catechol estrogens. This inhibition results in a decrease in the formation of 2-methoxyestradiol which acts as a strong antigenic and anticancer agent; furthermore, it can increase levels of procarcinogenic 4-hydroxyestradiol; both of these can contribute to the pathogenesis of estrogen-stimulated hormonal cancers [63].

Impact of B9/B12 Deficiency on Immune Cells

The roles of folic acid and B12 in DNA and protein synthesis and cell maintenance and proliferation are vital when looking at how deficiency

states can affect the immune system. Both cell-mediated immunity and humoral immunity are compromised in folic acid and B12 deficiency states and the effects on immune cells are varied and numerous. Supplementation of folic acid and B12 has been shown to improve immune response in both animal and human models in many studies [8, 64–69]. However, some studies have also reported that over-supplementation can also adversely affect immune function [64, 70, 71].

Monocytes/Macrophages

Monocytes are precursor cells for macrophages and dendritic cells and are located in the bone marrow where they are released into the bloodstream upon maturation. Differentiation of monocytes into macrophages occurs in tissues as a response to environmental stimuli such as cytokines and microbial products. Monocytes differentiate into either M1 (immune-active) or M2 (immune-suppressive) macrophages.

The status of folic acid plays a major role in the immune system. Pre-treatment with folic acid of cultured human monocytes has been shown to inhibit HCY-induced nuclear factor kappa B (NF-κB), which in turn is an important factor in the gene regulation of pro-inflammatory cytokines [65]. Patients with megaloblastic anemia, which is a clinical form of folate deficiency, display impaired immune responses affecting largely cell-mediated immunity. This condition, however, can be reversed with supplementation of folic acid [8]. In addition, folic acid is effective in reversing hyper-responsiveness of LPS-induced chemokine secretion from monocytes of patients with HHCY [72]. Chung et al. [73] noted that mononuclear cells from patients with HHCY show a greater expression of pro-inflammatory cytokines compared to monocytes from control subjects [73].

Mononuclear cells play a major role in the development of vascular diseases [74, 75] by attaching themselves to injured endothelium after vascular injury and supporting tissue remodeling in injury repair. In many vascular cell types, hyperlipidemia and oxidized LDL increase the expression of pro-inflammatory mediators which result in the formation of atherosclerotic plaques. Likewise, in murine monocyte cell line (RAW264.7) grown in a folate deficiency state, there were a decrease in intracellular folate levels, a reduced growth rate, and a two- to threefold increase in the expression of inflammatory mediators (e.g., IL-1-β, IL-6, TNF-α, and monocyte chemoattractant protein 1) at the RNA and protein level [76].

Vitamin B12 deficiency states can also affect the function of monocytes. In one study, the production of TNF-α was amplified in the spinal cord of B12-deficient rats [77]. Similarly, the synthesis of TNF-alpha was upregulated in macrophages of B12-deficient mice [78]. Furthermore, B12-deficient rats showed a decrease in IL-6 as a consequence of gp130 dysregulation. gp130 is a transmembrane glycoprotein. It is the founding member of the class of all cytokine receptors and stimulates demyelination in the central nervous system of mammals [79]. Vitamin B12 supplementation corrected both IL-6 and TNF-α deviations [69]. In addition, in a randomized controlled trial, vitamin B12 supplementation reduced inflammation and HCY levels in 30 healthy individuals [67]. In contrast, in 364 Saudi subjects, high serum vitamin B12 levels were associated with pro-inflammatory cytokines [80], while high levels of dietary folate were shown to negatively affect immune health in human subjects [70].

T Cells

$CD4^+$ T cells are important in providing an adaptive immune response to a wide variety of pathogens and a crucial component of immune protection. They play a central role in autoimmune disease, allergic reactions, asthma, and tumor immunity. $CD4^+$ T cells stimulate B cells to produce antibodies; induce and enhance microbicidal activity by macrophages; play a crucial role in the recruitment of neutrophils, eosinophils, and basophils to infection and inflammation sites; and orchestrate immune response through production of cytokines and chemokines

[15, 81]. Naïve conventional $CD4^+$ T cells differentiate into one of four (or possibly more) subsets of cells dependent on signaling molecules they encounter during antigen interaction, these being helper T (T_H)1, T_H2, T_H17, and induced regulatory T cells (iTreg). Two signals are usually required for naïve T cell activation, T cell receptor (TCR) and costimulation via accessory molecules such as CD28, CD80, and CD86, all of which are expressed on mature dendritic cells and several other antigen-presenting cells [15].

$CD8^+$ T cells, otherwise known as cytotoxic T cells, like $CD4^+$ T cells are generated in the thymus but differ in that they express a dimeric coreceptor. Cytotoxic T cells recognize peptides presented by major histocompatibility complex (MHC) class I molecules found on all nucleated cells. Cytotoxic T cells can kill infected or malignant cells in three different ways via the production of cytokines such as TNF-α and interferon gamma (IFN-γ), release of cytotoxic granules such as perforin and granzymes, and Fas/FasL interactions which trigger caspase cascades resulting in apoptosis.

Studies have shown that folate deficiencies can lead to a reduced resistance to infection by decreasing the ratio and proliferation of circulating T cells which respond to mitogenic activation [82]. Culturing activated T cells in folate-deficient media results in reduced T cell proliferation, cell cycle arrest in S phase, apoptosis, increased uracil levels in DNA, and reduced $CD8^+$ T cell proliferation which effectively increases $CD4^+/CD8^+$ ratio. Folate supplementation restored T cell proliferation and normal cell cycle, reduced DNA uracil content, and lowered $CD4^+/CD8^+$ ratio. Thus, folic acid deficiency may affect the immune system by reducing the capacity of $CD8^+$ T cells to proliferate in response to activation [83]. Furthermore, diets both low and excessively high in folate can cause defective cell cycle progression, persistent DNA damage, and impaired lymphocyte production [70]. Similarly, folic acid is crucial for the survival and differentiation of Treg cells although it is not necessary for the differentiation of naïve T cells into Treg cells [84].

Vitamin B12 plays an important role in immunity by facilitating production of T lymphocytes, maintaining the balance of $CD4^+/CD8^+$ ratio, and preserving lymphocyte subgroup count within a normal range. Several studies have been undertaken in patients with pernicious anemia who display decreased T cell count, increased $CD4^+/CD8^+$ ratio, and decreased NK cell activity. Vitamin B12 replacement therapy was successful in restoring all these imbalances [12, 85], although a later study by Watanabe et al. suggested that B12 replacement therapy does not show this [86].

Natural Killer Cells

NK cells are important effector lymphocytes belonging to the innate immune system. They derive their name from their capacity to respond to and directly kill pathogenic invaders through cytotoxic means. NK cells lyse target cells using cytokines such as INF-γ granzymes or perforin. NK cells are constantly circulating through the bloodstream as surveillant cells, and one of their primary roles is to combat viral infections through direct killing of infected cells and the recruitment of adaptive response. Both high and low folate status have demonstrated a negative impact on the function of these cells. Folic acid and B12 supplementation and deficiency have been shown in many studies to compromise hematopoiesis resulting in defective cell cycle function, DNA damage, and impaired function of NK cells [12, 20, 87–90]. Advanced aging is associated with impaired function of NK cells. In fact in aged rats an imbalance in B12 and folic acid altered NK cytotoxicity after just short-term dietary treatment [91], while in another study in humans, excessive folic acid intake in the elderly with low B12 status had adverse health outcomes including exacerbation of clinical anemia and cognitive impairment and increased methylmalonic acid and HCY plasma levels [92].

Conclusions

Vitamins B9 and B12 are dietary nutrients essential for proper maintenance of immune health and cellular function. They contribute to methylation reactions, cellular function and division, DNA

synthesis, repair, replication, and production of neurotransmitters. A dietary deficiency of B9 and B12 can lead to impairment of vital functions within the body including serine/glycine cycles and methylation and an increase in homocysteine levels. This in turn can cause immune dysfunction leading to pathogenesis of disease such as cardiovascular, kidney, and neurovascular diseases, osteoporosis, and cancer progression. There is much literary evidence to suggest that adequate dietary levels of B9 and B12 can act as preventative measures for inflammation, immune dysfunction, and disease progression.

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Vitamin B1, B2, B3, B5, and B6 and the Immune System

7

Kathleen Mikkelsen and Vasso Apostolopoulos

Contents

Key Points

- B vitamins are necessary for the proper functioning of the methylation cycle.
- A decrease in the methylation function leads to chronic neurological disorders.
- Thiamine, niacin, riboflavin, pantothenic acid, and pyridoxine have anti-inflammatory properties, and their deficiency leads to a number of immune disorders.

Introduction

Thiamine or niacin deficiency leads to gut epithelial tight junction defects, reduced antioxidant enzymes, and increased inflammation. Their supplementation gives rise to anti-inflammatory responses. Likewise, riboflavin has anti-inflammatory properties and enhances resistance and phagocytic activity against bacterial infections. Pantothenic acid has anti-inflammatory properties and restores gastrointestinal microbiome, and pyridoxine which influences cognition has anti-inflammatory and antibacterial properties, although at high concentrations in the body, it leads to a pro-inflammatory state. The link between vitamin B1, B2, B3, B5, and B6 with the immune system is discussed (Fig. 7.1) [1–5].

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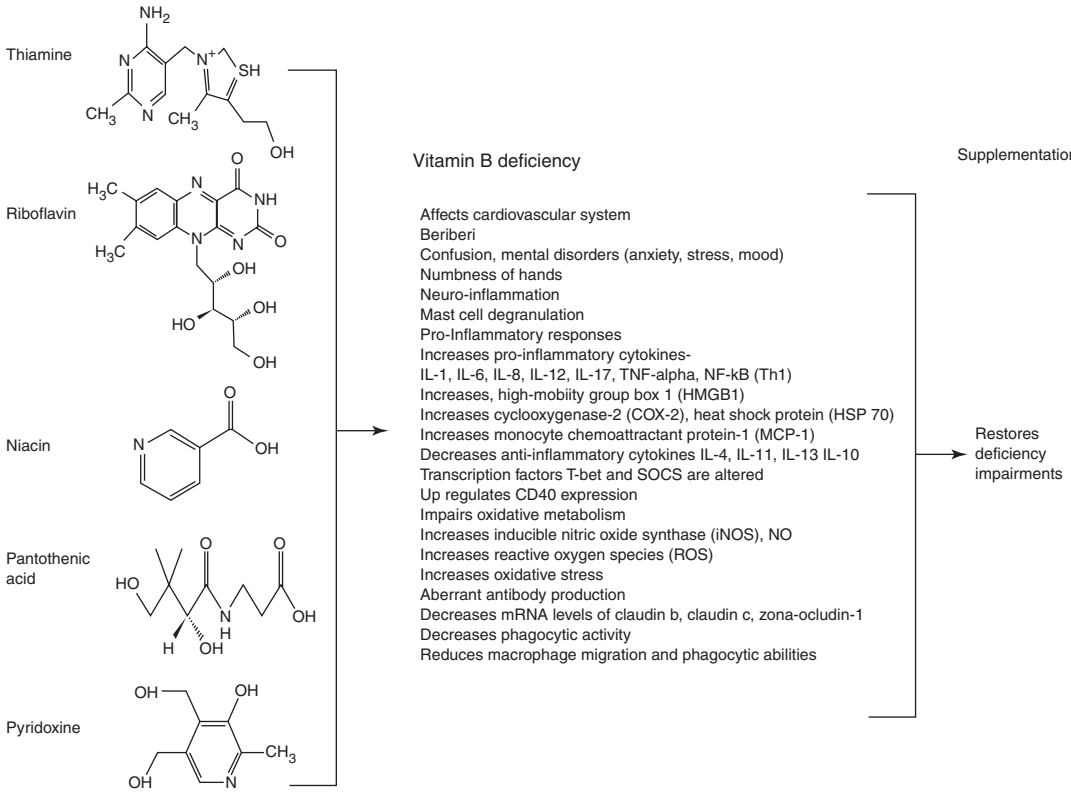

Fig. 7.1 Effects of thiamine, riboflavin, niacin, pantothenic acid, and pyridoxine deficiency on the immune system

Vitamin B1 (Thiamine) and the Immune System

Vitamin B1 (thiamine) is required by the body for the efficient breakdown of carbohydrates. It is present in an array of food products including grains, nuts, beans, yeast, and meat. Thiamine is used to boost the immune system and may help to reduce risk of type 2 diabetes, cardiovascular disease, aging-related disorders, eye disorders (poor vision, cataracts, and glaucoma), kidney disease, cancer, neurodegenerative diseases (Alzheimer's disease), and mental disorders (depression) [2, 5–9].

Thiamine deficiency results in beriberi (which affects the cardiovascular system and nervous system and causes numbness of feet and hands and confusion) and neuroinflammation. Thiamine supplementation usually resolves symptoms within weeks, and the disease may be prevented by balanced food intake and thiamine fortification [10]. In addition, lack of thiamine in the body impairs oxidative metabolism as a result of inadequate enzymatic activity, disruption of the blood-brain barrier, astrocyte dysfunction, reduced glucose metabolism, and chronic pro-inflammatory responses [11]. Numerous studies have been reported in recent years in order to understand the role of thiamine in the immune system. Thiamine deficiency in rats leads to increased mast cell degranulation and histamine levels in the thalamus in the early stages of Wernicke's encephalopathy, which is followed by an increase in astrocytes and macrophage cells in the brain stem [12]. Upon thiamine supplementation, histamine levels are reduced to baseline levels [13]. Thiamine deficiency in mouse models shows neurodegeneration, inadequate oxidative metabolism, inflammation, and inflammation-induced neuronal death [14]. In addition, deficiency of thiamine upregulates CD40 expression on microglial cells and CD40 ligand (CD40L)

expression on astrocytes which induce neuronal death [15]. CD40 and CD40L are co-stimulatory molecules which activate immune responses. In fact, in CD40L-knockout mice, the onset of thiamine deficiency-induced neuronal death is significantly delayed as well as the activation of microglial cells and endothelial cells [16]. Thiamine deficiency also increases inducible nitric oxide synthase (iNOS) levels in murine macrophages and microglial cells in the brain which is associated with oxidative stress and neuronal cell death [17]. Microglial cells stimulated with lipopolysaccharide (LPS) in vitro show characteristics of pro-inflammatory response. However, in the presence of benfotiamine (synthetic thiamine), iNOS, NO, cyclooxygenase-2 (COX-2), heat shock protein 70, interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are decreased, whereas, IL-10 is increased [18]. Hence, thiamine is an anti-inflammatory vitamin and inhibits the pro-oxidative activity of microglial cells which could have therapeutic potential for neurodegenerative disorders. Likewise, murine macrophage cell line RAW264.7 treated with LPS stimulates the expression of prostaglandin E2, thromboxane 2, prostacyclin, leukotrienes, NF-κB, iNOS, COX-2, and induce macrophage cell death, which all are inhibited in the presence of benfotiamine [19]. These data suggest that thiamine supplementation may be beneficial in the treatment of inflammatory disorders.

In thiamine-supplemented sheep, neutrophils are more effective in eliminating *Candida albicans* (candidacidal activity) compared to neutrophils from non-thiamine-supplemented sheep [20]. In addition, rats fed a thiamine-deficient diet are unable to eliminate *Escherichia coli* endotoxin as efficiently as compared to control rats [21]. Immune responses are also altered in thiamine-deficient lake trout where significantly increased levels of T cell-independent antibody responses and decreased T cell-dependent antibody responses compared to control trout are noted [22]. Thiamine deficiency also decreases the number of naïve B cells in Peyer's patches suggesting the important role of thiamine in humoral immunity [23]. These studies show that thiamine deficiency leads to aberrant antibody responses. Further, grass carp (*Ctenopharyngodon idella*) fed a thiamine-deficient diet shows decrease mRNA levels of claudin-b, claudin-c, claudin-3, zonula occludens 1 (ZO-1), and occludin which allow gut proteins to pass through the intestinal tight junctions resulting in systemic inflammation [24]. Thiamine deficiency also leads to decreased complement 3, hepcidin, liver-expressed antimicrobial peptides 2, IL-10, tumor growth factor beta (TGF-β), malondialdehyde and increased expression of IL-1, IL-8, interferon gamma (IFN-γ), TNF-α, and NF-κB in fish gills of grass carp [24]. Thus, thiamine deficiency leads to pro-inflammatory responses, disturbance of tight junction proteins, antioxidant enzymes, and NF-κB. In addition, in a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), it was shown that thiamine deficiency accelerated the development of EAE, with microglial activation and >140% increase in the number of infiltrating pro-inflammatory helper T (T_H)1 and T_H17 cells and chemokine CCL2 in the spinal cord [25]. In rats, daily injections of thiamine for 21 days reduce paw edema, thermal hyperalgesia, IL-1, and TNF-α in complete Freund's adjuvant-induced inflammation/arthritis. These data support the anti-inflammatory function of thiamine [26].

Immune dysfunction in patients with cancer who underwent surgery is well-documented and leads to low lymphocyte counts. Supplementation with vitamin B1, B6, and B12 shows marked improvement in lymphocyte counts and functionality (response to PHA and PPD) in a study in gastric cancer patients [27]. In addition, benfotiamine supplementation has anti-AGE (advanced glycation end products) properties possibly by restoring immune homeostasis in particular in diabetic patients [28]. It has been shown that patients with chronic tonsillitis have lower lymphocyte function and phagocytic activity which correlate with low thiamine levels [29]. It is likely that thiamine supplementation could improve immune response in such patients.

Vitamin B2 (Riboflavin) and the Immune System

Vitamin B2 (riboflavin) maintains healthy blood cells, boosts metabolism and energy levels, and is a powerful antioxidant. It is found in dairy products, vegetables, eggs, grains, and meat, and riboflavin deficiency leads to stomatitis, migraines, poor cognitive outcomes, depression, and personality disorders [2]. Riboflavin plays a role in oxidative metabolism and has anti-inflammatory properties. In fact, riboflavin deficiency in cultured macrophage cell line, RAW264.7, leads to increased levels of pro-inflammatory cytokines IL-1 and TNF-α, high-mobility group box 1 (HMGB1) protein, iNOS, NO, heat shock protein 72, and monocyte chemoattractant protein-1 (MCP-1), which are all reversed upon riboflavin supplementation [30]. Riboflavin supplementation also stimulates the anti-inflammatory cytokine IL-10. In addition, riboflavin-deficient cultured murine adipocytes show significantly increased expression of reactive oxygen species (ROS), IL-6, TNF-α, leptin, and NF-κB and reduced expression of adiponectin [31]. Such states would have consequences for insulin resistance and metabolic syndrome disorders. In addition, in active demyelinating lesions in patients with multiple sclerosis, there is extensive oxidative damage to astrocytes and macrophages which can be reversed by antioxidant enzymes as well as riboflavin. Indeed, riboflavin was shown to have anti-inflammatory and anti-oxidative effects in a clinical study of 197 patients with multiple sclerosis [32].

LPS treatment in co-cultures of adipocytes and macrophage cells leads to obesity-related inflammation, as reflected in the increased production of pro-inflammatory mediators, e.g., IL-1, IL-6, TNF-α, MCP-1, HMGB1, and reduced macrophage migration, which could be reversed with the addition of riboflavin [33]. The survival rate of mice receiving a lethal dose of LPS is approximately 10% which is increased to 80% in mice treated with riboflavin plus the amino acid valine. A clinical study in humans with sepsis and riboflavin supplementation is therefore warranted [34]. Likewise, infection of macrophages with *Staphylococcus aureus* stimulates a pro-inflammatory response, and supplementation with riboflavin would enhance phagocytosis and antioxidant enzymes and decrease pro-inflammatory cytokines, IL-1, IL-6, IFN-γ, TNF-α, and NO [35]. Additionally, zymosan treatment of peritoneal macrophages in mice induces high levels of HMGB1 which are significantly reduced with riboflavin supplementation [33]. In mice, zymosan-induced peritonitis is associated with high levels of metalloproteinase 9 (MMP-9), MCP-1, NO, IL6, IL-12, TNF-α, and IFN-γ. Riboflavin co-injection can decrease all these pro-inflammatory parameters [36]. Furthermore, riboflavin deficiency impairs macrophage phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 activity against *Listeria monocytogenes*, which is rapidly restored by riboflavin supplementation [37]. Thus, riboflavin deficiency impairs macrophage and adipose functionality, and its supplementation can alleviate associated pro-inflammatory status. Riboflavin supplementation can therefore be used to diminish inflammation as well as enhance macrophage phagocytic activity.

Excessive exposure to carbon tetrachloride (CCl4) induces liver toxicity. CCl4 was therefore injected in rats in order to induce hepatotoxicity, as a measure of altered hepatic enzymes, oxidative stress parameters, and pro-inflammatory cytokine TNF-α. Histopathologic analysis of rat livers showed that riboflavin supplementation reversed all these biochemical parameters [38]. In young grass carp, riboflavin-deficient diet corresponds to decreased levels of glutathione peroxidase activity, glutathione reductase, and lysozyme levels in the intestines. In addition, mRNA levels of anti-inflammatory cytokines (IL-10 and TGF-β) and tight junction proteins (occludin, ZO-1, claudin-b, and claudin-c) are decreased. Increased mRNA levels of pro-inflammatory cytokines (IL-1, IL-8, TNF-α, NF-κB, and the tight junction strand claudin-12) are noted as well [39].

Recently, it was shown that when the fibroblast NIH3T3 and the human embryonic kidney 293 (HEK293T) cell lines were cultured in riboflavin-deficient media, cell proliferation was

enhanced by over twofold. Likewise, tumor cells that were cultured in riboflavin-deficient media grew larger. The increased cell growth corresponded to lower p21 and p27 protein expression and higher cyclin D1 and CDK4 levels and cell cycle acceleration [40]. Thus, riboflavin is involved in tumor cell growth and may be a contributing factor for tumor development. Further research is required to understand the molecular mechanisms and cross-talk between riboflavin and cancer cells. As such, HepG2 human liver cancer cell line cultured in riboflavin-deficient media and analyzed by bioinformatics showed increased activity of Parkinson's disease-related pathways, e.g., endoplasmic reticulum stress and apoptotic process, whereas fatty acid and iron metabolism and oxidative phosphorylation were decreased [41].

In recent years, the identification of mucosal-associated invariant T (MAIT) cells and their ligands has revolutionized our understanding of the gut immune system and its role in immune homeostasis. T cells recognize antigenic peptides or lipopeptides in complex with the major histocompatibility complex (MHC) or CD1, respectively. MAIT cells, however, recognize antigenic bacterial vitamin B metabolites (riboflavin and folic acid produced by the gut microbial flora) in complex with the MHC-like molecule, MR1 [42]. This represents a novel mechanism by which the immune system has evolved to detect bacterial infections. Upon interaction of MAIT cells with MRI-riboflavin bacterial ligand, IFN-γ, TNF-α, and IL-17 are secreted to aid in the destruction of bacterially infected cells [32]. More recently, the role of MAIT cells has been described in diseases such as in *Helicobacter pylori* infection, type 1 diabetes, multiple sclerosis, and cancer [43]. A better understanding of the role of MAIT cells in disease may lead to alternative immunotherapeutic approaches for such diseases.

Clearly, riboflavin is an effective anti-inflammatory modulator that exerts protective effects against oxidative damage, enhances macrophage phagocytic activity, protects intestinal tight junction protein damage, and shows anti-tumor effects, and its metabolites are involved in MAIT cell stimulation. The role of riboflavin in health and disease is becoming evident, and further research is warranted to ascertain its mechanisms of action and how riboflavin can be utilized as a therapeutic agent.

Vitamin B3 (Niacin) and the Immune System

Vitamin B3 (niacin, nicotinic acid) is required in the body to convert carbohydrates into glucose (energy production), aid in the production of fatty acids and cholesterol, and repair DNA damage. Also, it is important for the proper functioning of the nervous and digestive systems and in keeping the skin, nails, and hair healthy. Niacin is found in whole and processed foods, tuna, meat, eggs, milk, yeast, and vegetables. Its supplementation is associated with lower lipid levels and lower cardiovascular risk factors linked to dyslipidemia. Although its deficiency is rare, low levels of niacin result in depression, disorientation, dementia, skin disorders, and pellagra [44]. In patients with unipolar depressive disorder, supplementation with niacin greatly improves depression, anxiety, and somatic symptoms [45].

Niacin is commonly prescribed for its anti-inflammatory effects in inflammatory disorders, particularly osteoarthritis, as well as for lipid-lowering effects. In fact, in an open-label study of 48 non-ST-elevated acute coronary syndrome patients, niacin supplementation decreased high-sensitivity C-reactive protein (hs-CRP) and cholesterol levels [46]. The differentiation of monocytes into macrophages in the presence of postprandial triglyceride-rich lipoprotein (TRLs) polarizes the differentiation into M1 pro-inflammatory macrophages. However, in the presence of niacin, monocytes differentiate into M2 anti-inflammatory macrophages [47]. Nicotinic acid receptor (GPR109A, HCA2) inhibits the breakdown of fats and prevents atherosclerosis and is used to reduce cholesterol levels. GPR109A is expressed on adipocytes and immune cells (neutrophils, macrophages, monocytes, dendritic cells). The presence of high doses of niacin results in anti-inflammatory effects in the brain,

gastrointestinal tract, skin, and vascular tissues [48]. Niacin also decreases neuroinflammation and has been considered as a potential therapeutic agent for neuroinflammatory diseases including Parkinson's disease and multiple sclerosis. Interestingly, in the early 1970s, large supplementation of nicotinamide and thiamine was used to arrest and reverse multiple sclerosis and repair damaged nerve cells. Activation of GPR109A by niacin leads to a marked decrease in neuroinflammation via adaptive protein beta-arrestins signaling [49], decreasing levels of pro-inflammatory cytokines (IL-6, TNF-α), MCP-1, and NF-κB and reducing monocyte chemotaxis [50]. Similarly, niacin inhibits IL-8 and leukotriene B4-induced chemotaxis of neutrophils [51]. In addition, niacin restores stem cell function through amelioration of mitochondrial dysfunction. Oral administration of nicotinamide in gestational diabetic rats reduces glucose levels, increases insulin levels, upregulates superoxide dismutase and catalase genes in the liver, and reduces oxidative burst activity in neutrophils [52]. These studies suggest that nicotinamide could be used as a therapeutic agent for gestational diabetes.

Vascular inflammation and endothelial dysfunction are induced by a periarterial carotid collar in rabbits. Feeding a niacin-rich diet to the rabbits reduces the levels of MCP-1 and vascular cell adhesion molecule-1 (VCAM-1), inhibits neutrophil recruitment and cyclic guanosine monophosphate production, and protects against hypochlorous acid-induced endothelial dysfunction and TNF-α-induced vascular inflammation [53].

Similarly to that noted with thiamine and riboflavin deficiency in young grass carp (*Ctenopharyngodon idella*), a niacin-deficient diet impairs intestinal mucosal immune function and gill immunity. Niacin-deficient diet results in decreased lysozyme and acid phosphatase activities, decreased production of anti-inflammatory cytokines (IL-10 and TGF-β), and increased mRNA expression of pro-inflammatory cytokines (IL-1, IL-8, IFN-γ, and TNF-α) in the intestines. In addition, niacin deficiency correlates to increased levels of ROS and malondialdehyde, decreased glutathione peroxidase activity, decreased glutathione reductase and lysozyme levels, decreased intestinal tight junction proteins (claudin-b, claudin-c, claudin-3, claudin-15, occludin, and ZO-1), and increased claudin-12 levels [54]. In rats, chemically induced inflammatory bowel disease results in high levels of angiostatin, endostatin, myeloperoxidase, IL-10, TNF-α, and vascular endothelial growth factor (VEGF). Niacin supplementation could reverse all these events and attenuate the severity of colitis. Of note, mepenzolate bromide, a GPR109A blocker, can inhibit the effects observed by niacin supplementation. Thus, niacin is effective in ameliorating chemically induced colitis in rats by decreasing the inflammatory milieu [55].

Airline pilots are highly exposed to ionizing radiation and therefore at a high risk of DNA damage. Eighty-two airline pilots were studied in regard to their vitamin B dietary intake and related it to the frequency of chromosome translocations as a biomarker of cumulative DNA damage. It was noted that niacin dietary intake in the form of whole grains but low in red and processed meat (but not from supplements or folate, riboflavin, vitamin B6, and B12) is associated with decreased chromosome translocation frequency [56]. Thus, niacin appears to be protective against DNA damage induced by ionizing radiation.

Exciting research places niacin in an excellent position for further research to determine its anti-inflammatory and DNA protective properties in disease amelioration. Nutritional supplementation using niacin may prove to be an effective alternative approach to alleviate disease.

Vitamin B5 (Pantothenic Acid) and the Immune System

Vitamin B5 (pantothenic acid, pantothenate) is present in all living cells and foods, but predominantly in yeast, liver, legumes, eggs, avocado, and fortified cereals. Pantothenic acid plays a pivotal role in metabolism of carbohydrates, fats, and protein for energy production and in the development of red blood cells, vitamin D_3, cho-

lesterol, phospholipids, amino acids, and fatty acids and helps to maintain a healthy neurological system. Its deficiency is rare; however, depression and respiratory infections are noted. Pantothenic acid becomes co-enzyme A, an enzyme required for the production of cortisol and acetylcholine; thus, its role in the normal development of the central nervous system is established [5]. Recently, in 3845 Iranian adults who completed a health questionnaire, it was noted that those on a high pantothenic acid diet were less likely to have psychological disorders (anxiety and depression) compared to those on other diets [57]. In another study, vitamin B intake including pantothenic acid correlates with lower bipolar disorder, depression, and increased overall mental health [58]. In rats, high doses of pantothenic acid reduce depressive symptoms in those kept under disturbed daily rhythm [59]. In addition, hopantenic acid, a homologue of pantothenic acid, reduces cognitive and anxiety disorders in patients with arterial hypertension and stroke [60]. Based on these findings, further research is warranted into using pantothenic acid or its homologues as alternative treatment modalities in people with mood disorders. Interestingly, pantothenic acid is significantly increased in marathon runners and soccer players but not body builders suggesting a role of aerobic activity in the metabolism of pantothenic acid. Hence, exercise could be used as a source of improving pantothenic acid levels which in turn would also have positive effects on mental health status of individuals [3].

Pantothenic acid has anti-inflammatory properties. In 908 subjects, higher pantothenic acid intake correlated to lower serum CRP concentrations [61]. Numerous disorders have been associated with deficiency in vitamin D_3, which alters the intestinal microbiome leading to reduced vitamin B production, including pantothenic acid. In particular, atherosclerosis and rheumatoid arthritis correlate with vitamin D_3 and pantothenic acid deficiency. Supplementation with pantothenic acid and vitamin D_3 in over 1000 patients resolved bowel symptoms by improving intestinal microbiome of vitamin B-secreting bacteria (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*), improved sleep, and significantly reduced pain and inflammation [62]. In addition, dexpanthenol, a derivative of pantothenic acid, is used for wound healing and in cosmetic and pharmaceutical products (ointments, lotions, shampoos, and lozenges). In mice, dexpanthenol significantly improved lung edema associated with lung injury, inhibited neutrophil accumulation in the lung, and reduced IL-6, TNF-α, malondialdehyde, and myeloperoxidase activity, as well as increased superoxide dismutase [63]. Likewise, in endometriosis, dexpanthenol reduced TNF-α, total oxidant status, and oxidative stress index [64]. In necrotizing enterocolitis, dexpanthenol attenuated intestinal injury (villus height, number of goblet cells, and histological score) and reduced superoxide dismutase, glutathione activities, and pro-inflammatory cytokine (IL-1 and TNF-α) production [65]. Hence, dexpanthenol has anti-oxidative and anti-inflammatory activities. Furthermore, pantothenol, an alcohol analogue of pantothenic acid, is used as a moisturizer and in wound healing. Pantothenol also inhibits proliferation of malaria parasite, *Plasmodium falciparum*, and may be used as a drug against malaria [66].

Vitamin B6 (Pyridoxine) and the Immune System

Vitamin B6 constitutes six compounds, pyridoxine, pyridoxal (comprising three forms in tissues), pyridoxamine, pyridoxal-5-phosphate (the main active form), 4-pyridoxic acid (the main excretory form), and pyridoxine hydrochloride (the main form in supplements). Pyridoxine is present in an array of foods such as organ meats, legumes, cereals, fruits, and vegetables [67]. Pyridoxine is essential for the synthesis of hemoglobin and for the production of neurotransmitters such as serotonin, dopamine, and melatonin. Pyridoxine is involved in the formation of other neurotransmitters (norepinephrine, GABA, glutamate, and endorphin) and is necessary for the regulation of neuronal activities and integrity [68]. However, it in high concentrations may cause

sensory nerve damage. Clinical deficiency of pyridoxine is uncommon; however, symptoms include nervous system disorders (depression and confusion), seborrheic dermatitis, microcytic anemia, reduced immune responses, and increased inflammation [68]. Infants feeding an autoclaved formula (in which pyridoxine is damaged) presented with hyperirritability and seizures which could be reversed with pyridoxine supplementation [69]. In a case report, a 36-year-old male with chronic alcoholism presented with seizures, which were resistant to anti-epileptic drugs but disappeared following pyridoxine administration [70]. Pyridoxine deficiency has also been implicated in autoimmune disorders, such as type I diabetes [71], rheumatoid arthritis, and multiple sclerosis [8].

Pyridoxine deficiency decreases antibody production and increases IL-4 levels. In addition, mice feeding a pyridoxine-deficient diet show altered T cell responses such as inhibition of T cell proliferation, reduction in IL-2 levels, increase in IL-4 levels, and alteration in the expression of transcription factors, *T-bet* and SOCS-1 d [72]. Likewise, dietary pyridoxine deficiency in young grass carp (*Ctenopharyngodon idella*) results in reduced anti-inflammatory cytokines (TGF-β, IL-4, IL-10, IL-11, and IL-13) and increased pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IL-15, and IL-17) [73]. Furthermore, pyridoxine shows antibacterial properties against biofilm-embedded *Staphylococcus aureus* and *Staphylococcus epidermidis* [74]. It is clear that pyridoxine has anti-inflammatory and antibacterial properties. However, high dietary supplementation of pyridoxine can cause sensory neuropathy, high IFN-γ secretion, and cytokine imbalance toward Th1 [75].

Conclusions

Thiamine, niacin, riboflavin, pantothenic acid, and pyridoxine have anti-inflammatory properties and play a crucial role in the immune response, and their deficiency leads to a number of disordered states.

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Zinc and the Immune System

8

Nour Zahi Gammoh and Lothar Rink

Contents

Key Points

- Zinc is an important trace metal which is required for growth and survival. It is involved in many biochemical and physiological processes and is a cofactor of numerous enzymes as well as being a structural component of many proteins.
- Zinc deficiency affects every organ of the body. Howv this influences health depends on how severe the deficiency is.
- Zinc Homeostasis and zinc signaling are regulated by controlling zinc uptake from extracellular space and redistribution between intracellular compartments via zinc transporters (ZIPs and ZnTs), or by releasing zinc from storage proteins such as MTs and albumin.
- Free zinc pools play an important role in signal transduction. There are three types of zinc signals characterized according

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to the time scale by which they emerge and how long they last for; zinc flux, zinc wave, and homeostatic zinc signal.
- Zinc deficiency causes adverse effects on the immune system such as autoimmune disorders, allergies, increased susceptibility to infections, thymic atrophy, and cancers.
- Zinc deficiency induces immune dysfunctions in both innate and adaptive immunity. It causes reduced lytic activity, impaired signaling, altered cytokine production, reduced proliferation, among many other dysfunctions.
- Zinc deficient individuals have higher susceptibility to infectious diseases.
- Many of the negative outcomes of zinc deficiency can be reversed by restoring adequate zinc levels through zinc supplementation.

Introduction

Zinc is the second most abundant trace metal in the body after iron and is essential for growth and survival. Since its importance for human health has been highlighted by Prasad and colleagues in the 1960s [1], studies continue to reveal the involvement of zinc in many biochemical and physiological processes. It is an important cofactor of over 300 enzymes [2], and as a structural component, it plays a major role in protein stabilization. Furthermore, it has been shown that zinc is essential for many other critical functions such as cell growth, differentiation, DNA synthesis, and RNA transcription.

Major impacts on human health involving zinc are mainly due to its deficiency, as acute toxicity is very limited. Virtually all organs are influenced by zinc deficiency (ZD), albeit this influence depends on how severe the zinc deficit is. Severe ZD can occur under certain circumstances, for example, in patients suffering from acrodermatitis enteropathica (AE), a rare and untreated lethal genetic disorder that causes zinc malabsorption. Clinical manifestations of severe ZD include impaired growth, bullous pustular dermatitis, testicular hypofunctions, neurosensory disorders, mental retardation, diarrhea, delayed wound healing, and recurrent infections due to an impaired immune system. Those who suffer from moderate ZD, mainly due to nutritional deficiency, also experience similar clinical manifestation yet not as acute as those suffering from severe ZD [3]. Milder forms of ZD are harder to recognize; however, with the aid of experimental models, it has been shown that inducing mild ZD in a group of male volunteers resulted in a decreased serum testosterone level, oligospermia, decreased natural killer cell lytic activity, decreased interleukin (IL)-2 activity of T helper cells, decreased serum thymulin activity, hyperammonemia, hypogeusia, decreased dark adaptation, and decreased lean body mass. These results confirm that even mild ZD adversely affect human health [4].

Even though zinc plays a vital role in all systems and organs, the focus of this chapter will be on the influence of zinc on the immune system. Zinc contributes vastly to the host defenses by maintaining membrane barriers as well as being crucial for the normal development and functioning of all immune cells. ZD induces thymic atrophy and lymphopenia and affects both innate and adaptive immune responses. It impairs macrophage functions such as phagocytosis, intracellular killing, and cytokine production; host defenses by neutrophils and natural killer (NK) cells; as well as the proliferation, antibody secretion, and cytokine production of B and T cells [5]. This chapter will attempt to provide an up-to-date comprehensive overview of the involvement of zinc in modulating the immune response, as well as a brief run-through of some nutritional aspects and how zinc homeostasis is maintained within the body.

Zinc in Nutrition

Recommended Intake

Due to the significance of zinc for human health, a landmark decision was made by the US Food and Nutrition Board in the 1970s to establish

recommended daily allowances (RDAs). Currently, the recommended intake of zinc is 11 mg/day and 8 mg/day for adult males and females, respectively [6]. While in other countries, these RDAs may differ slightly, for example, the German Society of Nutrition's recommendation comprised of 10 mg/day and 7 mg/day for adult males and females, respectively [7]. Both the World Health Organization (WHO) and the European Food Safety Authority (EFSA) take into consideration the inhibitory effect of dietary phytate on zinc absorption when setting the RDAs. WHO categorizes diets according to their potential absorption efficiency of zinc, per phytate-zinc molar ratio, into three groups, high (<5), moderate (5–15), and low (>15) zinc bioavailability [8], whereas EFSA provides different zinc reference recommendations for diets containing phytate intake levels of 300, 600, 900, and 1200 mg/day [9]. On the other hand, there is a recommended upper limit for zinc intake. The National Institutes of Health (NIH) in the USA has stated that adults must not consume over 40 mg/day [10], while the Scientific Committee on Food (SCF) in Europe has set the Tolerable Upper Intake Level (UL) of 25 mg/day [9]. Those UL guidelines are there to avoid chronic high intake of zinc which can lead to severe neurological or immunological impairment due to copper deficiency.

There are several factors that influence zinc levels in the body, such as age, sex, weight, and phytate content of the diet. As mentioned above, one of the important factors influencing zinc bioavailability and absorption through the intestines are the nondigestible plant ligands, such as phytates, which strongly bind zinc and reduce its absorption. The bioavailability of zinc varies considerably in different food groups. Zinc from animal sources is more bioavailable compared to that from plant sources, which explains how diets that consist mainly of plant products such as those consumed in developing countries, or by vegans and vegetarians, may lead to lower zinc intake and eventually ZD [11]. Table 8.1 gives a few examples of certain foods and their zinc content. Additionally, there are certain groups of people who have an increased requirement for zinc and at higher risk of developing ZD. These groups include infants and children, adolescents, pregnant and lactating women, and the elderly [12]. Oral zinc supplements are readily available but not all offer the same zinc bioavailability. Zinc bound to amino acids such as aspartate, cysteine, and histidine shows the highest absorption concentration, followed by zinc chloride, sulfate, and acetate, whereas zinc oxide shows the lowest bioavailability [13].

Table 8.1 Examples of different food groups and their zinc content [14]

Food (preparation method and serving size)	Zinc (mg) per serving
Oysters, cooked, breaded, and fried (85 g)	74.0
Beef chuck roast, braised (85 g)	7.0
Crab, Alaska king, cooked (85 g)	6.5
Beef patty, broiled (85 g)	5.3
Breakfast cereal, fortified with 25% of the DV for zinc (18.75 g)	3.8
Lobster, cooked (85 g)	3.4
Pork chop, loin, cooked (85 g)	2.9
Baked beans, canned, plain or vegetarian (12.5 g)	2.9
Chicken, dark meat, cooked (85 g)	2.4
Yogurt, fruit, low fat (227 g)	1.7
Cashews, dry roasted (28 g)	1.6
Chickpeas, cooked (12.5 g)	1.3
Cheese, Swiss (28 g)	1.2
Oatmeal, instant, plain, prepared with water (28 g)	1.1
Milk, low-fat or non-fat (240 ml)	1.0
Almonds, dry roasted (28 g)	0.9
Kidney beans, cooked (12.5 g)	0.9
Chicken breast, roasted, skin removed, ½ breast	0.9
Cheese, cheddar or mozzarella (28 g)	0.9
Peas, green, frozen, cooked (12.5 g)	0.5

Assessing Zinc Status

According to the WHO, it is estimated that ZD affects about one-third of the world's population, with estimates ranging from 4% to 73% across subregions. ZD is responsible for approximately 16% of lower respiratory tract infections, 18% of malaria, and 10% of diarrheal diseases [15]. To develop proper nutritional intervention programs, it is important to establish a reliable marker for any particular nutrient. Despite the clear impact

of ZD, there is no defined marker for mild to moderate ZD. This is primarily due to the lack of a specialized zinc storage system in the body, such as with iron. The total zinc content in the human body (70 kg) amounts to 2–4 g, with a plasma concentration of 12–16 μM. This plasma pool is small, consisting of 0.1% of body zinc, yet rapidly exchangeable and mobile [2].

In recent years, scientists have been striving to establish an accurate marker to indicate zinc levels in the body. One of the most commonly used markers is blood, plasma, or serum zinc concentration. It is, so far, the best available option to recognize ZD. Despite some suggested lower cutoff values set by the 2nd National Health and Nutrition Examination Survey in the US population [16], assessing mild and moderate ZD using plasma or serum concentration is not very reliable. Zinc concentrations fluctuate throughout the day, mainly due to food and water consumption. Additionally, low serum zinc can occur in several conditions, such as during acute infection and inflammation [17], stress [18], myocardial infarction [19], cirrhosis, nephrotic syndrome and renal insufficiency [20], and hormonal treatment or pregnancy [21]. In contrast, conditions that lead to intrinsic or extrinsic hemolysis of blood cells may result in very high serum zinc levels due to the high concentration of zinc present intracellularly. Consequently, serum or plasma zinc concentration should be interpreted with care, taking into consideration the complete clinical picture of each person.

Other tools that can be used to evaluate zinc status are using food frequency questionnaires, which can help evaluate zinc content in the diet and assess the risk of ZD. Food frequency questionnaires are good tools to study the intake of certain nutrients but they need to be standardized and normalized for each population. Furthermore, the prevalence of nutritional stunting (height-for-age) can be used as an indicator for ZD; taken together with serum values, it can be a good marker to evaluate those at risks of ZD [22]. Other biosensors that can also be utilized to measure zinc are fluorescent probes that have high affinity and selectivity to zinc ions. However, these sensors are usually unstable and inconvenient when it comes to preparation, storage, and in vivo transference, thus limiting their practicality [23]. In conclusion, currently there is no perfect tool to measure zinc, but by using a combination of tools, zinc status of individuals and populations can be assessed to a relatively accurate degree, until more reliable and accurate tools are designed.

Zinc Homeostasis

Most of the body's zinc is found intracellularly. 30–45% of intracellular zinc is found in the nucleus, about 50% is found in the cytoplasm and other cellular organelles, and the remaining zinc is located in the cell membrane. Only the free form of zinc can participate in cellular signaling. There are several mechanisms that regulate free zinc homeostasis and thereby zinc signaling. These mechanisms comprise of controlling zinc uptake from extracellular spaces, redistribution between different intracellular compartments, or through the reversible oxidative release of free zinc from storage proteins [24].

There are two major protein families that are responsible for transporting zinc into and out of the cells and redistributing it within cellular compartments. The first group of transporters is referred to as ZIPs (Zrt/Irt-like proteins), which are responsible for transporting zinc into the cytosol from either extracellular space or from intracellular compartments. There are 14 ZIP transporters, designated as solute carrier family SLC39A1-A14. The second group of 10 transporters is referred to as ZnT (Zinc transporters), which are designated as SLC30A1-A10. They generally transport zinc out of the cytosol into extracellular space or intracellular organelles such as zincosomes. Zincosomes are vesicles that can sequester high levels of zinc [24–26]. Each transporter shows specific tissue distribution and cellular localization. Mutations in these proteins have been linked to many conditions. An overview of ZIPs and ZnTs can be seen in Table 8.2.

There are some proteins that bind metal ions such as zinc and act as a cellular buffering system. Metallothioneins (MTs) come on top of the list of those proteins. MTs are cysteine-rich 6–7 kDa proteins that can form a complex with

Table 8.2 Overview of ZIPs and ZnTs [32–34]

	Transporter (gene name)	Tissue distribution	Cellular localization	Disease-related condition
ZnT Transporter	ZnT1 (SL30A1)	Ubiquitous	Plasma membrane	Embryonic lethal (mice)
	ZnT2 (SL30A2)	Mammary gland, prostate, retina, pancreas, small intestine, kidney	Endosomal/lysosomal/secretory vesicle, ER in breast cancer cells, zymogen, granules in pancreatic acinar cells, inner mitochondrial membrane of mammary cells	Nursing mothers produce zinc-deficient milk (human)
	ZnT3 (SL30A3)	Brain, testes, pancreas	Synaptic vesicle	Seizures, learning deficit, memory loss (all in mice)
	ZnT4 (SL30A4)	Ubiquitous, predominant in mammary gland, placenta, prostate, brain, kidney	Endosomes/lysosomes, cytoplasmic vesicles, trans-Golgi network	Lethal milk (mice)
	ZnT5 (SL30A5)	Ubiquitous, predominant in heart, placenta, pancreas, prostate, ovary, testes, small intestine, thymus, bone	Golgi, unknown Vesicles (isoform 1 in mice and humans) Plasma membrane (isoform b in humans)	Bone, poor growth, heart failure (all in mice)
	ZnT6 (SL30A6)	Ubiquitous, predominant in brain, lung, intestine	*trans*-Golgi network, unknown vesicles	Alzheimer (human)
	ZnT7 (SL30A7)	Ubiquitous, predominant intestine, stomach, prostate, retina, pancreas, testes, muscle	Golgi, unknown vesicles	Prostate cancer, low adiposity, diet-induced diabetes (all in mice)
	ZnT8 (SL30A8)	Pancreas, thyroid, adrenal gland, testes	Secretory granule	Diabetes (mice and human)
	ZnT9[a] (SL30A9)	Ubiquitous	Cytoplasm, nucleus	Unknown
	ZnT10 (SL30A10)	Brain, retina, liver	Early/recycling endosomes or Golgi apparatus	Parkinsonism and dystonia with hypermanganesemia, polycythemia, hepatic cirrhosis (human)

(continued)

Table 8.2 (continued)

	Transporter (gene name)	Tissue distribution	Cellular localization	Disease-related condition
ZIP Transporter	ZIP 1 (SLC39A1)	Ubiquitous, testes	Plasma membrane, intracellular vesicles	Prostate cancer, neurodegeneration (human)
	ZIP 2 (SLC39A2)	Ubiquitous, testes	Plasma membrane	Prostate cancer (human)
	ZIP 3 (SLC39A3)	Ubiquitous, mammary cells, testes	Plasma membrane, lysosomes	Neurodegeneration and allergic airway inflammation (mice), prostate cancer (human)
	ZIP 4 (SLC39A4)	Gastrointestinal tract, kidney hippocampal neurons	Plasma membrane, Apical surface of enterocytes, lysosomes	AE, pancreatic cancer, liver cancer, gliomas (human)
	ZIP 5 (SLC39A5)	Pancreas, kidney, liver, stomach, intestine, eye	Plasma membrane, Basolateral surface of enterocytes	Zinc-induced acute pancreatitis (mice)
	ZIP 6 (SLC39A6)	Ubiquitous	Plasma membrane	Breast, pancreatic, cervical, prostate cancer, neuroblastoma (human)
	ZIP 7 (SLC39A7)	Ubiquitous	ER, Golgi, intracellular vesicles	Breast cancer (human)
	ZIP 8 (SLC39A8)	Ubiquitous, T cells, erythroid, testis	Plasma membrane, Lysosomes, mitochondria	Inflammation, sepsis, breast cancer, osteoarthritis, cadmium-mediated toxicity in lung (human)
	ZIP 9 (SLC39A9)	Ubiquitous	Plasma membrane, *trans*- Golgi	Breast and prostate cancer (human)
	ZIP 10 (SLC39A10)	Brain, liver, erythroid, kidney	Plasma membrane	Breast cancer, follicular B lymphoma (human)
	ZIP 11 (SLC39A11)	Testes, digestive system	Nucleus, Golgi	Unknown
	ZIP 12 (SLC39A12)	Brain, lung, testes, retina	Plasma membrane	Unknown
	ZIP 13 (SLC39A13)	Ubiquitous	Intracellular vesicles, Golgi	Spondylocheirodysplastic Ehlers-Danlos syndrome (human)
	ZIP 14 (SLC39A14)	Ubiquitous, liver	Plasma membrane	Asthma, inflammation, colorectal cancer (human), growth retardation (mice)

[a]ZnT9 is thought to have no zinc transport functions, because it lacks an essential histidine constituting the intramembranous zinc-binding site and has not been shown to interact with other ZnT transporters. It acts as nuclear receptor coactivator and is renamed as GAC63 (GRIP1-associated coactivator 63)

up to 7 zinc ions. They bind up to 20% of the intracellular zinc, which can be readily released. There are four different MT classes. MT-1 and MT-2 are ubiquitous throughout the body. Their main function is to maintain cellular zinc homeostasis and chelate heavy metals to reduce cytotoxicity and lower their intracellular concentrations. Due to their reactive oxygen species (ROS) scavenging properties, they help protect against several types of environmental stress. MT-3 and MT-4 expression are restricted to a cell type-specific pattern. MT-3 is predominantly found in the brain and MT-4 is primarily located in stratified epithelial tissues [27, 28].

Another prominent zinc-binding protein is albumin. Albumin binds ~80% of plasma zinc and acts as a major zinc transport protein in plasma. Although zinc does not dissociate from albumin as readily as other zinc-binding proteins, it still has a considerable rapid exchange kinetics and contributes to the homeostasis of free zinc in the plasma [29]. Furthermore, S100 protein family, which consists more than 20 members that are distributed in a cell-specific and tissue-specific manner, have diverse functions that range from calcium buffering, enzyme activity and secretion modulation, involvement in apoptosis and transcription, among other functions. Those proteins are also regulated by zinc, whereby several members show high-affinity towards zinc such as S100B, S100A1, S100A2, S100A3, S100A5, S100A7, S100A8/9, S100A12, and S100A16. S100A8/9 forms the Zn^{2+}-binding heterodimeric protein calprotectin, which is expressed in high levels in myeloid cells. S100 proteins are reviewed in more detail elsewhere [30]. Moreover, α2-macroglobulin (A2M) is another zinc-binding protein which is an inhibitor of matrix metalloproteases (MMPs). A2M plays a protective role during inflammation against overproduction of pro-inflammatory cytokines [31]. This role will be discussed in more detail later.

Zinc Signaling

During proliferation or under certain stimulatory conditions, the small free zinc pool is altered. In fact, intracellular zinc ions are never free; they are usually loosely bound to amino acids, phosphates, or other low molecular weight ligands, but the term "free zinc" is established to underline its availability and flexibility. Unlike the majority of cellular zinc, which is tightly bound to proteins, this labile pool of readily available zinc plays an important role in signal transduction.

The impact of zinc on the immune system is partly due to its important role as a signaling ion, participating in immune cell signal transduction which results in modulation of gene expression.

Zinc signals are characterized into three types according to the time scale by which they emerge and how long they last for [35–37]:

1. Zinc flux: occurs within less of a minute after stimulation which suggests a role for zinc as a second messenger. This signal does not last long and it is independent of changes in gene expression and synthesis of proteins such as ZIPs and ZnTs. Zinc flux is observed after lipopolysaccharides (LPS) or phorbol-12-myristate-13-acetate (PMA) induction in myeloid cell lines. Another source of zinc that can be rapidly released is zinc bound to MTs.
2. Zinc wave: a zinc signal which lags for a few minutes after stimulation but continually increases for at least an hour. In mast cells, cross-linking of the high-affinity immunoglobulin E receptor, Fc-epsilon receptor I (FcεRI), induced a zinc wave that was dependent on calcium influx.
3. Homeostatic zinc signal: develops later and can last for one or more days. This signal involves altering the gene expression of zinc binding proteins and transporters required for zinc metabolism and regulation, which leads to the alteration of intracellular zinc following the initial stimulus. This type of signal initiates permanent changes in zinc levels which are required for cellular processes such as maturation and differentiation of myeloid and dendritic cells.

As mentioned previously, zinc signaling involves several mechanisms. The first of which is regulating zinc transporter activity through phosphorylation by kinases. The second is the release of protein-bound zinc via the production

of redox-active substances such as nitric oxide (NO) from inducible nitric oxide synthase (iNOS) or ROS from NADPH oxidase. The third is the regulation of the expression levels of zinc binding proteins or transports as mentioned above. The role of zinc in signaling will be discussed in the following sections and is also reviewed extensively elsewhere [37, 38].

Effect of Zinc on the Immune System

The immune system is a highly proliferating organ that is influenced by the availability of certain nutrients. It is a well-documented fact that ZD is often accompanied by several immune-related disorders such as autoimmune disorders, allergies, increased susceptibility to infections, thymic atrophy, and cancers. Zinc not only influences every cell in the immune system but also affects the expression levels of hundreds of genes.

The immune system consists of two parts, the innate and the adaptive immunity. Innate immunity comprises the first line of defense, which includes granulocytes, monocytes/macrophages, dendritic cells (DC), mast cells, and natural killer (NK) cells as cellular components and several proteins, including the complement system. Cells of the innate immune system are the first to encounter invading pathogens and eliminate them. The hallmark of the innate immune system response is inflammation. However, the innate immune system lacks memory and cannot provide long-lasting protection against reinfection with the same microorganisms. On the other hand, the adaptive immune system is subdivided into humoral immunity (B cells) and cellular immunity (T cells). These cells recognize specific antigens and can differentiate into effector and memory cells. Studies have revealed how zinc influences each of those cell types, which will be discussed in more detail in the next section.

Innate Immunity

The innate immune response is the first line of defense against invading pathogens. This response is rapid yet unspecific against these pathogens. Innate immune cells are activated by pathogen-associated molecular patterns (PAMPs), which are conserved molecular structures found in groups of pathogens that can be recognized by conserved receptors on cells. Those receptors are designated as pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs). In human, there are 10 different TLRs that allow cells to recognize and distinguish different molecules present on a variety of pathogens. For example, TLR1 detects peptidoglycan (gram-positive bacteria), TLR2 detects zymosan (fungi), and TLR4 detects LPS (gram-negative bacteria) [39]. When PAMPs bind to their specific receptors, a signaling cascade is activated which leads to the initiation of a variety of antimicrobial events such as, phagocytosis, cytokine and chemokine secretion, direct killing of the pathogens, or antigen presentation to cells of the adaptive immune system. Furthermore, secreted cytokines and chemokines facilitate cellular communication between cells of the innate immune system and those of the adaptive immune system [24].

The main TLR signal transduction pathway is mediated by the recruitment of the myeloid differentiation primary response gene (MyD) 88 adapter. MyD88 recruits the interleukin-1 receptor-associated kinase (IRAK) family. This, in turn, leads to the phosphorylation of IRAK and further recruitment of the tumor necrosis factor receptor-associated factor 6 (TRAF6) and transforming growth factor [TGF]-β activated kinase 1 (TAK1). Then, TAK1 triggers activation of nuclear factor kappa B (NF-κB) pathway through inhibitor of NF-κB kinase (IKK) and also triggers the mitogen-activated protein kinase[MAP]-kinase (MKK) enzymes resulting in activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase pathways. This transcriptional regulation results in the production of cytokines and other pro-inflammatory processes [40]. On the other hand, there is a MyD88 independent signaling pathway that involves Toll-interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon (TRIF). After the activation of MyD88, the

receptor is endocytosed and then binds to TRIF and to TRIF-related adaptor molecule (TRAM). This leads to the delay of NF-κB signaling and the activation of IFN regulatory factor 3 (IRF3); this signaling pathway results in the secretion of IFN-β [41]. The IFN bind type 1 IFN receptor (IFNR) which activates the Janus kinase (JK)-signal transducer and activator of transcription (STAT) pathway, consequently inducing the expression of CD40, CD80, and CD86, which are required for T-cell interaction [42]. Activation of NF-κB and JAK-STAT pathways induces the expression of iNOS [43, 44]. NO which is a product of iNOS is an important mediator of inflammation and is required for the removal of invading microorganisms and cancer cells [45]. Zinc influences those signaling molecules either directly or indirectly via phosphatases, kinases, and redox metabolism [46].

From the moment a cell surface receptor binds to its ligand to the induction of gene expression, there are several processes that mediate signal transduction. Molecules in the signaling cascade undergo phosphorylation via protein kinases. In immune cells, there are several residues that are acceptors of phosphate residues. The most common is tyrosine, but there are also serine and threonine residues. Phosphorylation by kinases can be removed by antagonizing phosphatases, balancing the extent of phosphorylation inside the cells. Phosphorylation of target residues can either lead to activation or inactivation of that molecule [47]. Zinc plays a role in the regulation of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Studies have revealed the inhibitory effect of zinc on several PTPs, even at the very low concentration found intracellularly [48]. Furthermore, protein kinase C (PKC) enzymes are also important for signaling in immune cells. It has been reported that PKCs are zinc metalloenzymes. Zinc is an important structural component of PKCs [49]. The inhibition of PKC activity when zinc is chelated might not only be due to the structural requirement of zinc but also because the translocation of PKC between membrane and cytoskeleton is zinc-dependent [50, 51].

One of the major signaling pathways in innate immune cells is the NF-κB pro-inflammatory pathway. It regulates the genes controlling apoptosis, cell adhesion, proliferation, tissue remodeling, immune responses, inflammatory processes, and cellular stress responses. Consequently, it influences the expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, IL-8, and MCP (monocyte chemoattractant protein)-1. NF-κB is one of the most versatile regulators of gene expression [52]. The NF-κB B protein family in mammalian cells consists of five members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). Different NF-κB complexes are also formed from homo and heterodimers. Non-active NF-κB complexes are typically found in the cytoplasm, where they are bound to and silenced by a family of inhibitory proteins known as inhibitors of NF-κB (IκBs). There are several of those inhibitors: IκBα, IκBβ, IκBγ, IκBε, and Bcl-3. Additionally, p100 and p105 also function as IκBs-like proteins which inhibit NF-κB-subunit dimeric partner, p50 and p52, respectively. Phosphorylation of IκBs by IκB kinases (IKK) results in the activation of NF-κB, whereby NF-κB is translocated to the nucleus where it can induce targeted gene expression [53].

Several studies have investigated the influence of zinc on NF-κB signaling; however, conflicting results make it difficult to agree on one conclusion. Therefore, the cell types and zinc concentrations used or chelators must be taken into consideration when results are evaluated. For instance, a study by Haase et al. has revealed that zinc is necessary for the activation of LPS-induced NF-κB signaling pathway in monocytes, where chelating zinc with membrane-permeable zinc chelator TPEN (N,N,N0,N0-tetrakis-(2-pyridyl-methyl) ethylenediamine) completely blocked this pathway [54]. Yet, more studies support the role of zinc as a negative regulator of NF-κB signal transduction. One of the major inhibitory mechanisms relies on how zinc affects the expression of protein A20. A20 is a zinc-finger protein that is recognized as an anti-inflammatory protein which also negatively regulates tumor necrosis factor receptor (TNFR) and TLR-initiated NF-κB pathways. During TNFR signaling, A20 is able to deubiquitinate receptor interacting protein 1 (RIP1), which prevents its interaction with NF-κB essential modulator IKK. It also inhibits TLR signaling by

removing polyubiquitin chains from TRAF6 [55]. However, the deubiquitinase activity of A20 remains unchanged by zinc chelator [24]. Additionally, zinc supplementation was able to downregulate inflammatory cytokines by decreasing gene expression of IL-1β and TNF-α through upregulation of mRNA and DNA-specific binding for A20, subsequently inhibiting NF-κB activation [56].

In addition to A20, there is another zinger-finger protein that plays a role in zinc-mediated signaling, peroxisome proliferator-activated receptor (PPAR) alpha. PPARα expression is induced by zinc, similar to A20. It inhibits the binding of NF-κB to the DNA, leading to the downregulation of pro-inflammatory cytokines and adhesion molecules [57].

The following sections describe the influence of zinc on each innate immune cell type as well as several mechanisms utilized by the innate immune system to protect the body against invading microorganisms.

Granulocytes

Granulocytes are the first cells to be recruited to the site of infection. There are three types of granulocytes; neutrophils, eosinophils, and basophils. Neutrophils are also referred to as polymorphonuclear neutrophils (PMN) and are the most abundant cells of the immune system. After pathogen invasion or inflammation, PMNs migrate to the infected tissue via adhesion and chemotaxis. Once they reach there, they perform several tasks such as phagocytosis, degranulation and release of antimicrobial proteins, secretion of cytokines, and finally release of neutrophil extracellular traps (NETosis). After phagocytosis, engulfed pathogens are killed using ROS. This process is known as oxidative burst and is primarily mediated by the activity of the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [58].

Zinc is essential for the proper function of granulocytes. During ZD, granulocytes show marked impairment in functioning. Zinc homeostasis should always be kept balanced, as ZD and zinc excess are both detrimental to granulocyte functions. Studies have shown that under ZD conditions, PMNs have reduction in phagocytic activity, oxidative burst granule release, NETosis, and chemotaxis [59, 60].

Neutrophil granules contain antimicrobial compounds used against invading pathogens. It has been shown that treating human neutrophils with zinc chelator TPEN resulted in reduced release of granules. It suggests that a zinc-dependent mechanism is required for the mobilization of granules from neutrophils [59].

Under normal conditions, cells produce ROS during cellular respiration. However, excessive production of ROS and the decreased rate of its neutralization and removal lead to an imbalance between oxidants and antioxidants which results in oxidative stress [61, 62]. Accumulation of those free radicals leads to cell and tissue damage. In order to neutralize and remove excess ROS, cells have endogenous non-enzymatic and enzymatic antioxidant defense mechanisms. One of the antioxidant enzymes is superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radical into the less harmful O_2 and H_2O_2. Zinc is a cofactor of the Cu/Zn-SOD enzyme. Additionally, it has been demonstrated that zinc deprivation may lead to increased NADPH oxidase-dependent ROS production, as zinc is a known inhibitor of NADPH oxidase activity [56]. In contrast, excessive zinc concentrations (10 mmol/L) inhibit ROS-generating capacity [63].

Alternatively, PMNs can kill pathogens by releasing neutrophil extracellular traps (NETs). These NETs are composed of DNA, chromatins, and granular proteins to capture and kill microorganisms. Chelating zinc using TPEN leads to the reduction of NET formation. The ROS-dependent signal transduction resulting in NETosis requires zinc signals as an essential component [60]. Moreover, calprotectin, which has been mentioned previously, is also released in very high concentrations during NETosis, where it is either incorporated into NETs or in the surrounding fluids [29].

Monocytes and Macrophages

Monocytes are an important component of the innate immune system. They migrate to tissues and differentiate into macrophages. There are several subtypes of macrophages displaying a

heterogenous tissue-resident cell population. These subtypes exhibit various functions including phagocytosis or pinocytosis, antigen presentation, and secretion of pro-inflammatory cytokines that together mediate the immune response.

Numerous studies have provided evidence that zinc is essential for the differentiation and normal function of monocytes and macrophages. However, results vary considerably depending on the experimental model used. Different cell lines, varying zinc concentrations, and incubation times are some of the examples of variable experimental setups that can produce inconsistent outcomes. For instance, zinc has been shown to increase by twofold the secretion of pro-inflammatory cytokines in monocytes. This enhancing effect appears to be concentration-dependent as demonstrated in human peripheral blood mononuclear cells (PBMC) treated with zinc which directly induced, in high concentrations, the secretion of interferon-gamma (IFN-γ), IL-1β, IL-6, and sIL-2R [64, 65]; in contrast, it also showed inhibitory effects on other stimulants inducing these cytokines [66, 67]. ZD augmented the effect of LPS and led to the secretion of large amounts of IL-1β, whereas high zinc concentrations diminished monokine secretion by suppressing the LPS-induced monocytic response involving inhibition of cyclic nucleotide phosphodiesterase (PDE) activity and expression. This resulted in the increase of cellular cyclic guanosine monophosphate (cGMP) which, in turn, suppressed LPS-induced tumor necrosis factor-alpha (TNF-α) and IL-1β production in human monocytes [68–70].

During 1, 25-dihydroxyvitamin D3 (1,25 D3)-mediated differentiation of $CD34^+$ hematopoietic stem cells into monocytic lineage, free intracellular zinc concentrations decrease. This might be due to the diminished expression of ZIP proteins and increased expression of zinc-binding proteins S100A8 and S100A9. Consequently, less zinc is taken up from extracellular spaces and more zinc is sequestered [71]. Similarly, incubating cells with TPEN or in zinc-depleted media enhanced 1,25 D3-mediated differentiation. Hence, establishing a low-zinc environment favors monocyte development. This can be seen during hypozincemia found in acute-phase response, suggesting a role of zinc in systematic signaling and the promotion of monocyte development [72].

Moreover, a role of zinc in macrophage polarization has been recently explored by Dierichs et al. [73]. Macrophages polarize into M1 or M2 phenotypes upon activation by environmental stimuli. IFN-γ in combination with LPS, for example, induces the polarization of macrophages into pro-inflammatory M1 cells, while IL-4 induces M2 polarization. Zinc supplementation in murine cell lines revealed an inhibitory effect on M2 polarization. This would implicate zinc in M2-related diseases such as allergic asthma and cancers. However, the M1 phenotype was promoted both during zinc-supplemented and ZD conditions which could be due to the lack of more distinct markers for macrophage phenotypes.

Furthermore, zinc-deprived mice had increased levels of IL-1β and IL-6 after LPS injection [74]. Macrophages derived from MT-KO mice showed impairment in phagocytosis, antigen presentation, and cytokine production. This indicates that MTs are crucial for functioning macrophages, and since MTs are key players in zinc homeostasis, it would suggest that zinc does play a crucial role in this regard [75]. Zinc supplementation has also been shown to affect the phagocytotic capacity in canine peripheral blood phagocytosis. There was a remarkable increase in phagocytic capacity of peripheral blood monocyte-rich cells. In the same study, zinc could also stimulate the production of TNF-α which consequently enhanced phagocytosis [76].

As for the signaling pathways in monocytes, TLR4 triggered with LPS is the most frequently investigated pathway. When LPS binds to TLR4, a zinc flux occurs. This leads to the inhibition of dephosphorylation of MAPKs and phosphorylation of IKKα/β. Subsequently, NF-κB is translocated into the nucleus where it induces the expression of pro-inflammatory cytokines [54]. Zinc chelation with TPEN in murine macrophages abrogated the activation of ERK1/2, IKKβ, MKK3/6, and IκB. Additionally, chelating zinc caused the accumulation of IRAK1 in the cytosol. However, the phosphorylation and ubiquitination

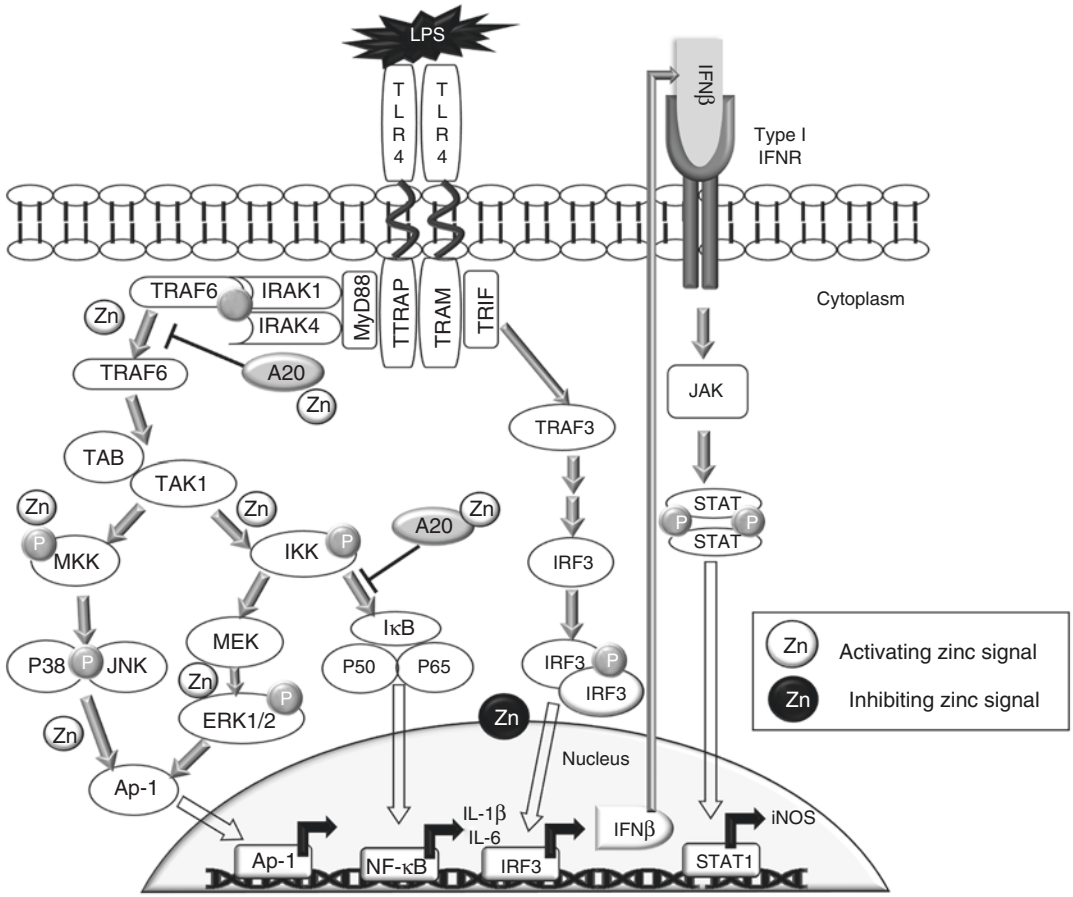

Fig. 8.1 Influence of zinc on TLR4 signaling pathway. Zinc supports phosphorylation of IKK so that NF-κB can be translocated to the nucleus. It is also required for the activation of kinases ERK1/2, IKK, MKK, and IκB. In contrast, zinc acts as an inhibitor of NF-κB signaling pathway by upregulating mRNA and DNA-specific binding for A20, which is a zinc finger protein that negatively regulated NF-κB signaling. Zinc also influences TRIF-mediated signaling pathway. Zinc differentially affects TLR4 signaling pathways on multiple levels, adapted from [36]. See text for further details

of IRAK1 were not affected in the initial zinc flux upon LPS-TLR4 activation. Degradation of IRAK1 is required for the release of phosphorylated IKKs into the cytoplasm. The IKKs in the cytoplasm are able to degrade IκB and P105 and thereby activate NF-κB and ERK signaling [77, 78]. Furthermore, it has been observed that LPS-stimulated murine macrophages produce more NO under zinc deficient conditions. Zinc chelation increases LPS-induced NO production by upregulating iNOS expression. Additionally, it is suggested that zinc regulates the TRIF signaling pathway by affecting IRF3 either through its nuclear phosphorylation, its translocation into the nucleus or the retention of its phosphorylated form [46]. Figure 8.1 summarizes how zinc influences TLR4 signaling pathways.

Moreover, there are several ligands for TLR that induce an increase of intracellular zinc in murine macrophages and primary human monocytes. Those ligands include Pam3CSK4 (TLR1/2), Listeria monocytogenes (TLR2), flagellin (TLR5), FSL-1 (TLR6/2), ssRNA40 (TLR7), and inhibitory oligonucleotides (ODN) 1826 (TLR9) [46, 54]. Additionally, stimulation of monocytes with monocyte chemoattractant protein (MCP-1), TNFα, insulin, and Pam3CSK4 also increased intracellular zinc [46].

In addition to studies investigating the influence of zinc supplementation and chelation on macrophages, further experiments have revealed a role of ZIP transporters in regulating the inflammatory response. This role was illustrated on several occasions. For example, ZIP8 expression was shown to be upregulated after stimulation of macrophages with LPS, which resulted in the increase of intracellular zinc. Zinc served as a negative regulator of the NF-κB signaling pathway by suppressing IKK activity in monocytes and macrophages [79]. Most recently, another ZIP transporter has been implicated in regulating macrophage function. ZIP10 was identified as the only ZIP transporter whose expression significantly decreases after stimulation of bone marrow derived-macrophages with LPS. In a *Zip10* knockout mouse model, it was revealed that macrophage survival during an inflammatory response depends on the zinc influx mediated by ZIP10. Without ZIP10, the resulting zinc deficiency triggered apoptosis. Additionally, inadequate intracellular zinc in *zip10*-knockout mice stimulated with LPS led to the reduction of stimulated macrophage which consequently lowered levels of serum inflammatory cytokines, prevented subsequent liver damage, and lowered mortality [80]. These studies suggest that certain transporters play an important role in modulating macrophage function in response to inflammatory stimuli.

Mast Cells

The influence of zinc on mast cells (MCs) has not been investigated thoroughly. However, there are some knowledge of the role of zinc in the signaling of MCs. MCs have immunoglobulin (Ig) receptors that detect immune complexes or opsonized pathogens. An example is the Fcε receptor (FcεR) that after binding induces degranulation, whereby antimicrobial granules are released. These granules contain high amounts of zinc, which most likely cause zinc intoxication of pathogens when released [81]. Zinc was also observed to be important for the degranulation process, as it mediates the translocation of granules to the plasma membrane. Some studies show that triggering FcεR induces a zinc wave. This zinc signal triggers the phosphorylation of ERK1/2 and JNK1/2 and the translocation of NF-κB to the nucleus, leading to the induction of pro-inflammatory cytokines. Inhibiting the zinc wave by incubating MCs with zinc chelator TPEN led to the suppression of signaling cascades and block of IL-1β and TNF-α release, while zinc supplementation conferred prolonged signaling [82].

Natural Killer Cells

NK cells are a subset of lymphocytes that make up 15% of human peripheral blood lymphocytes. They are part of the innate immune system because they do not express antigen-specific receptors or develop memory cells at the same time as B and T cells. Their main role is to target and kill virus-infected cells and tumor cells. They are able to recognize the downregulation of the major histocompatibility complex (MHC)-I molecules (mainly HLA-C) on the surface of infected cells and tumor cells via specialized ligands, the killer inhibitory receptors (KIR). When MHC-I is downregulated, the lack of KIR engagement leads to the activation of NK cells and induction of cytotoxic mechanisms. Furthermore, NK cells are engaged in antibody-dependent cellular cytotoxicity (ADCC). Whereby they express a range of invariant activating receptors such as Fc receptor for IgG which allows NK cells to interact with antibodies that are bound to surface antigen, thus initiating killing processes [83].

Zinc is crucial for NK cell development and cytotoxic function. Numerous studies have shown how ZD impairs NK cell function in humans [84, 85]. This was also observed after zinc chelation in vitro [84]. Yet this decrease of NK activity may be due to diminished stimulation by T cell-secreted IL-2 which is decreased during ZD. On the other hand, zinc supplementation increases the number of NK cells in whole blood [86]. This might be due to the enhancing effect zinc has on the differentiation and proliferation of $CD34^+$ progenitor cells towards NK cells. Differentiation of $CD34^+$ cells was impaired in elderly individuals; however, zinc supplementation led to partial correction [87].

Additionally, KIR binds zinc and requires zinc for recognition of HLA-C on target cells [88]. It

is particularly needed for the multimerization of KIR [89]. During ZD, impaired interaction with HLA-C may lead to unspecific killing. This is probably offset by reduced NK cell activity.

NK cells, like other immune cells, regulate the immune response by secreting cytokines. One important cytokine is IFN-γ, which is enhanced during zinc supplementation [86]. IFN-γ is important for the activation and maturation of many cells, which will be discussed later in the chapter.

Dendritic Cells

Dendritic cells (DC) are an important part of the innate immune response and they link the innate and adaptive immune system. They are phagocytic immune cells acting as professional antigen-presenting cells (APC) that express high levels of antigen-presenting MHC molecules. They can activate antigen-specific T lymphocyte, thereby initiating the adaptive immune response. They help maintain peripheral tolerance by regulating the activity of T effector cells and by inducing the immunosuppressive regulatory T lymphocytes (Tregs) [90]. In addition to their function as APCs, DCs also secrete several important cytokines, and like monocytes, they express PRRs such as TLRs on their cell surfaces that enable them to recognize pathogens [91].

Intracellular zinc fine-tunes DC maturation and functions. It has been observed that stimulation of murine DCs with LPS leads to downregulation of zinc importer ZIP6 and upregulation of zinc exporters via TLR4, together resulting in reduced intracellular free zinc and increased DC maturation. Similarly, chelating zinc with TPEN triggered DC maturation, indicated by increased levels of MHC expression [92]. MHC II molecules are located in lysosomal and endosomal compartments in immature DCs. Transport of these molecules from compartments is inhibited by zinc. Therefore, reduced intracellular zinc would enable translocation of MHC II molecules to the surface and facilitate antigen presentation and subsequent interaction with components of the adaptive immune system [93].

Membrane Barriers

Zinc may contribute to the host defense by maintaining the membrane barrier structure and function. This is essential in lung and intestine tissues that become constantly subjected to a range of pathogens and noxious agents. Several studies have investigated the influence of zinc depletion and supplementation on the permeability of the endothelial cell barrier. The intestinal epithelium barrier is composed of intercellular junctional complexes between neighboring cells that provide a continuous seal around the apical region of the cells. These complexes consist of several units, including the tight junctions (TJ) and adherens junctions (AJ) that form circumferential zones of contact between adjacent cells. E-cadherin is the main transmembrane adhesion molecule localized at the AJ and its binding to β-catenin is fundamental for appropriate AJ organization. Zinc depletion disrupted the TJ and AJ through several mechanisms. One way ZD affected structural proteins was the enhancement of degradation of E-cadherin and β-catenin [94]. This was also seen in zinc-deprived airway epithelial cells, where there was accelerated proteolysis of E-cadherin and β-catenin leading to increased leakage across the monolayer of upper and alveolar lung epithelial cultures [95]. ZD may induce uncontrolled neutrophil migration through the disrupted junctional complexes by inducing chemokine production. Exacerbated inflammation may develop and lead to mucosal damage which further contributes to intestinal and lung disease. On the other hand, zinc supplementation has been seen to preserve and restore membrane function and structure [94]. Heiliger et al. also demonstrated how zinc binds to the extracellular domain of N-cadherin and modulates N-cadherin-mediated adhesion in excitatory synapses of the central nervous system. The role of zinc in synaptic transmission and as an endogenous neuromodulator has already been investigated. But it was revealed in this study that increased extracellular concentration of zinc in the synaptic cleft reduced N-cadherin adhesion. This resulted in structural changes such as those responsible for synaptic plasticity and long-term potentiation. ZD might stabilize N-cadherin, leading to the impairment of those structural changes and reduce plasticity, which may explain, among other causes, why ZD rodent animal models exhibit memory and learning impairments [96].

Zinc is also an integral part of the epidermal and dermal tissues, where it acts as a stabilizer of cell membranes and as an essential cofactor in numerous transcription factors and enzymes. This includes the zinc-dependent matrix metalloproteinases that enhance autodebridement and keratinocyte migration during wound repair. Moreover, zinc confers resistance to epithelial apoptosis through cytoprotection against ROS and bacterial toxins possibly through antioxidant activity of the cysteine-rich metallothioneins [97]. Hence, ZD may result in delayed wound healing, particularly in the elderly with impaired nutritional status. Delayed wound healing in the elderly constitutes a major clinical and economic challenge, especially as the aging population grows [98]. Topical zinc therapy has shown promising results in enhancing wound healing due to the effect zinc plays in reducing the incidents of superinfections and necrotic material by augmenting local defense systems and collagenolytic activity, as well as promoting epithelialization of wounds [97]. Thus, utilizing topical zinc treatments to support wound healing provides a therapeutic advantage and enhances quality of life.

Peptidoglycan Regulation Proteins (PGLYRPs)

Zinc is also involved in the bactericidal activity of peptidoglycan recognition proteins (PGRPs or PGLYRPs). Those innate immunity pattern recognition molecules have effector functions and are expressed in either PMN molecules or in the skin, eyes, salivary glands, throat, tongue, esophagus, stomach, and intestine. Along with other antimicrobial peptides, PGLYRPs protect the body from pathogen at the first line of exposure [99]. The activity of PGLYRPs against Gram-positive and Gram-negative bacteria is dependent on free zinc [100].

Nutritional Immunity

Nutritional immunity refers to the competition for resources between host and pathogen. It is a concept that has been originally established for iron and now involves several trace metals including zinc. Pathogens also require zinc for survival and propagation within the host. The host utilizes several mechanisms to enable it to compete for zinc and achieve a zinc-limited environment for pathogens. However, some pathogens have developed ways to surpass those mechanisms [101, 102].

On a systematic level, free zinc in plasma is markedly reduced to limit its availability to invading pathogens. This mechanism involves secretion of inflammatory cytokines such as IL-6 that upregulate the expression of ZIP14 and MTs in hepatocytes, thereby accumulating zinc in the liver [103]. Furthermore, zinc concentrations can be altered on an extracellular level through the release of some antimicrobial peptides from the S100 family. Several cell types secrete different peptides. Keratinocytes secrete S100A7 that can kill *Escherichia coli* by sequestering zinc [104]. While neutrophils, as mentioned previously, secrete calprotectin, which can inhibit the growth of *Staphylococcus aureus* by sequestering zinc as well [105]. On an intracellular level, macrophages have evolved two opposing strategies to kill phagocytosed pathogens. Macrophages can deprive *Histoplasma capsulatum* of zinc by reducing the phagosome zinc content [106]. On the other hand, they kill *Mycobacterium tuberculosis* by intoxicating it with excess amounts of zinc and copper [107]. See Fig. 8.2 for summary.

However, the aforementioned antimicrobial strategies in innate immune cells that are primarily focused on depriving the pathogen of zinc are not invincible. Some pathogens have developed defense strategies against some of those mechanisms. For instance, *M. tuberculosis* utilizes Zn-effluxing ATPase CtpC to neutralize the toxic effects of zinc in a human-derived macrophage model of infection [108]. Furthermore, *Neisseria meningitidis* uses a high affinity zinc uptake receptor ZnuD which allows it to escape NET-mediated nutritional immunity [109]. It also responds to zinc deprivation by expressing CbpA, which is an outer membrane protein that acts as a receptor for calprotectin and enables *N. meningitides* to acquire zinc bound to calprotectin. *N. meningitidis* effectively undermines an important defense mechanism used by the host and utilizes it in its favor [110]. *Yersinia pestis* utilizes the siderophore yersiniabactin (Ybt) as a zincophore,

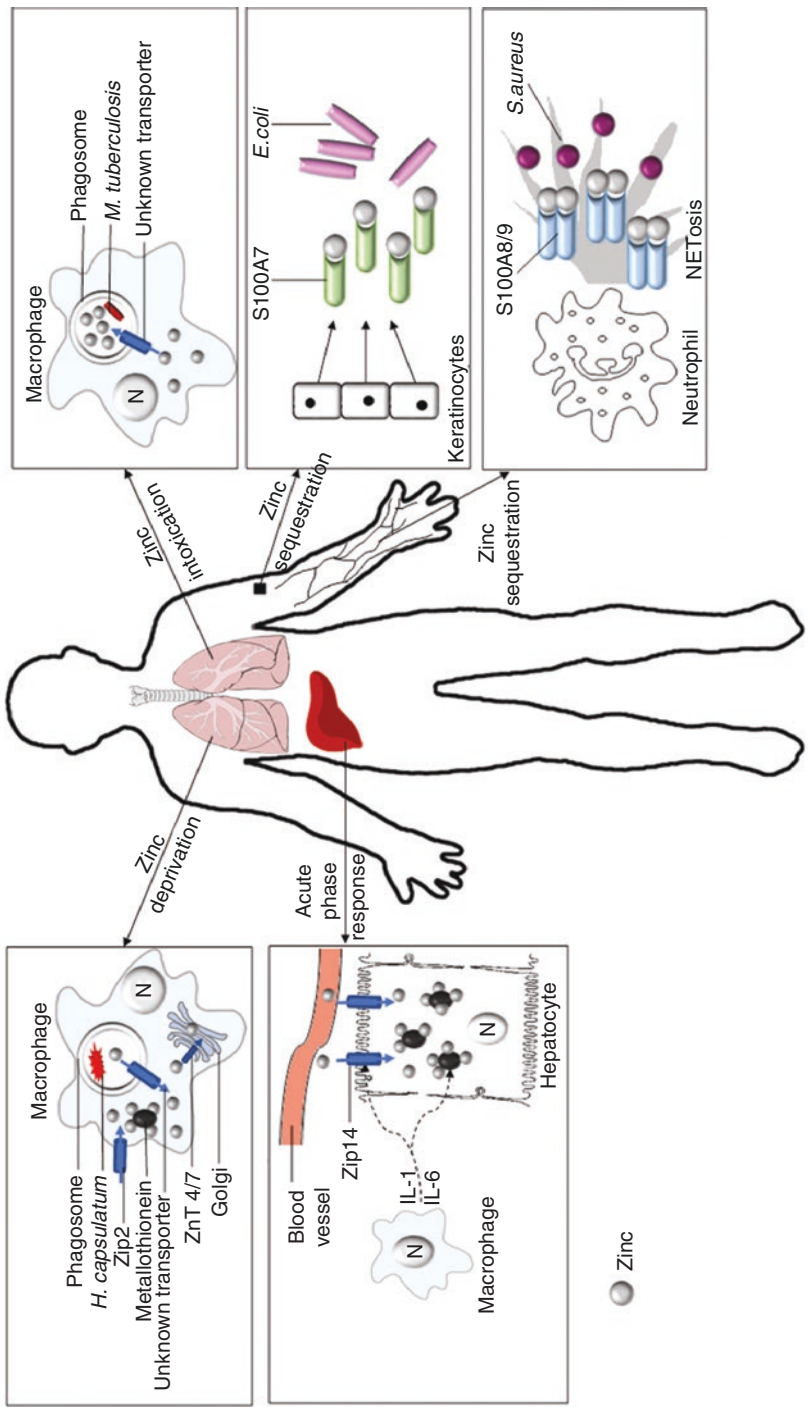

Fig. 8.2 Nutritional immunity and zinc. The body employs different strategies to avoid invasion by different microorganisms. Each strategy is used for a specific target. See text for details

and ZnuABC, which is the high-affinity zinc transporter found in bacteria and fungi, to acquire zinc and develop a lethal infection in a septicemic plague mouse model [111]. *Salmonella typhimurium* can also overcome calprotectin-mediated zinc chelation by expressing ZnuABC, thereby allowing it to compete with commensal bacteria and thrive in the inflamed gut [29].

Low zinc is associated with an impaired immune system and poor prognosis in conditions such as sepsis. Zinc status of septic patients is correlated to survival rates, septic scores, and other markers linked to sepsis. Low serum zinc correlates with recurrent sepsis episodes, increased chance of organ dysfunction, and a higher risk of mortality [112]. Nevertheless, zinc supplementation during the course of an infection in patients has not produced very promising outcomes. The reason behind this could be the counteractive effect of supplemented zinc to the immune system's efforts to limit zinc accessibility to pathogens, which in turn can promote bacterial growth rather than reduce it. Furthermore, a report using porcine model of sepsis revealed decreased zinc-binding capacity during sepsis due to reduction of zinc-binding proteins such as albumin; this suggests that even harmless doses of zinc can result in zinc toxicity and cause adverse effects [113]. Prophylactic zinc administration, so far, has shown positive outcomes in animal models, resulting in reduced bacterial load, decreased levels of circulating inflammatory cytokines, and enhanced survival rate [114–116]. However, unlike experimentally induced sepsis in animal models, it is hard to predict sepsis in humans, thus prophylactic zinc supplementation cannot be administered in time to confer the protective capacity of zinc. Therefore, administering zinc during an acute infection such as sepsis must be considered carefully in light of the evidence provided.

In contrast, zinc lozenges at concentrations of ≥75 mg/day reduced the duration of common cold symptoms in healthy individuals [117]. However, administration of zinc during an infection must be done with caution. It may assist creating a zinc-rich microenvironment that is favorable for pathogen development. Moreover, it may interfere with efforts carried out by the innate system to reduce free zinc. In this context, it would be interesting to refer to a study where the effects of nutrition on mice infected with a virus or bacteria were observed. The study revealed that nutrient deprivation increased the survival rate of bacterium-infected mice while it reduced the survival rate of virus-infected mice [118]. Therefore, further research is necessary to understand the effect of nutrient supplementation during infections and how the immune system adapts to cellular stress while allowing cells to access required nutrients.

Adaptive Immunity

The adaptive immune system consists of two main lymphocyte subsets: T and B lymphocytes. In contrast to cells of the innate immune system, T and B cells have T-cell receptors (TCR) and B-cell receptors (BCR) that are able to recognize specific antigen. This specific recognition leads to clonal expansion of the specific subset and the initiation of the adaptive immune response. Some of these activated lymphocytes persist as memory cells even after infection has cleared. Thus, when the same antigen is encountered, the adaptive immune system responds quickly and more efficiently, hereby preventing reinfection or lead to swift eradication of that pathogen. Furthermore, when B cells become activated, they develop into immunoglobulin-secreting plasma cells; hence, they play an essential role in humoral immunity. As for activated T cells, they express either cytotoxic or helper functions depending on which T-cell subset is activated. T cells are part of the cell-mediated immunity.

Altered zinc homeostasis influences both humoral and cell-mediated immunity either by affecting lymphopoiesis, cytokine secretion or indirectly by influencing the stimulation of cells of the innate immune system.

T Cells

T cells are subdivided into either $CD8^+$ cytotoxic T lymphocytes (CTLs) or $CD4^+$ T helper cells

(Th). CTLs, as the name implies, recognize and eliminate viral-infected cells and tumor cells. Th cells promote functions of other immune cells. Th cells are subdivided into Th1, Th2, Th17, and Tregs and their ratios are finely balanced to maintain proper immune function. Furthermore, some further Th subsets were described such as Th9, Th22, and follicular helper T cells (Tfh), which are so far not clearly defined as definitive lineages or activation state [119, 120]. Th1, Th2, and Th17 cells antagonize each other. Th1 cells promote cell-mediated immunity by secreting IFN-γ and IL-2 that mainly activate macrophages and dendritic cells, while Th2 cells promote humoral immunity by promoting production of antibodies by B cells via IL-4. Th17 cells promote the recruitment of neutrophils and regulate cell-mediated immune response against extracellular pathogens. They produce several pro-inflammatory cytokines such as IL-17A. Tregs play a role in suppressing the immune cells and inducing tolerance [72].

The thymus is the lymphoid organ where T-cell maturation occurs. Studies have reported thymus atrophy and size reduction during ZD in human children [121] and animals in vivo [122]. This is caused by glucocorticoids, which are chronically elevated in ZD mice and enhanced apoptosis of pre-T cells [123, 124]. This results in the reduction of mature T-cell numbers. Moreover, reduction of T cells is also caused by impaired thymulin activity. Thymulin is a thymic peptide hormone that is essential of T-cell differentiation and function and it requires zinc for its activity. In models of mild ZD in humans, diminished thymulin activity and decreased number of T cells were observed [125].

It has been shown that during ZD, cytokine production by Th1 cells is decreased, whereas no influence was seen in cytokine secretion from Th2 cells. This led to a net-shift of the Th1/Th2 ratio towards Th2 resulting in altered immune function [126]. A shift towards Th2 immunity may lead to an increased risk of developing allergic immune responses. Furthermore, differentiation of Th1 cells is modulated by intracellular zinc levels via the upregulation of IFN-γ, T-box transcription factor (T-bet), and IL-12 receptor beta 2 subunit (IL-12Rb2) after TCR stimulation by concanavalin A (Con A) and treatment with 5 μM zinc. These cytokines are important for differentiation of naïve $CD4^+$ T cells towards Th1 subsets, thus highlighting the importance of zinc for Th1 differentiation [127].

The effects of zinc on Treg cells have been investigated as well. In zinc-supplemented mixed lymphocyte culture (MLC), Treg cells were induced and IFN-γ production was reduced [128]. Zinc modulated the allergic immune response by enhancing the number of Tregs after PBMC were challenged with grass pollen [129]. Furthermore, zinc enhanced the capacity of TGFβ1 to induce Treg cells [130]. Additionally, zinc administration induced Tregs in experimental autoimmune encephalomyelitis (EAE) which is an experimental model for multiple sclerosis. In the same experiment, moderate zinc doses resulted in the reduction of Th17 number and diminished EAE score [131].

Th9 cells have been identified relatively recently as a new Th subset. They are major contributors to autoimmune diseases. Although they are poorly characterized as they have no known lineage-defining transcription factors, Th9 cells can be identified by IL-9 expression. IL-9 is a cytokine that plays several contrasting roles. It can either upregulate or downregulate immune functions. A recent study from our group has revealed for the first time the influence of zinc supplementation on Th9 cells in an in vitro model of graft versus host disease (GVHD), namely, mixed lymphocyte culture (MLC), as well as on resting T cells in PBMCs. Zinc supplementation exhibited opposite effects in activated versus resting T cells. IL-9 expression and secretion were reduced in MLC under zinc-supplemented conditions, indicating dampened Th9 expression. On the other hand, zinc supplementation induced Th9-mediated IL-9 expression and secretion in PBMCs. Thus, this suggests that zinc can mitigate against the unwanted alloreaction in GVHD in vitro as well as promoting the beneficial pro-inflammatory T-cell response in resting T cells which is required to defend the body against invading pathogens [132]. Figure 8.3 provides an overview of how zinc influences subpopulations of T helper cells. As for CTLs, ZD is associated with

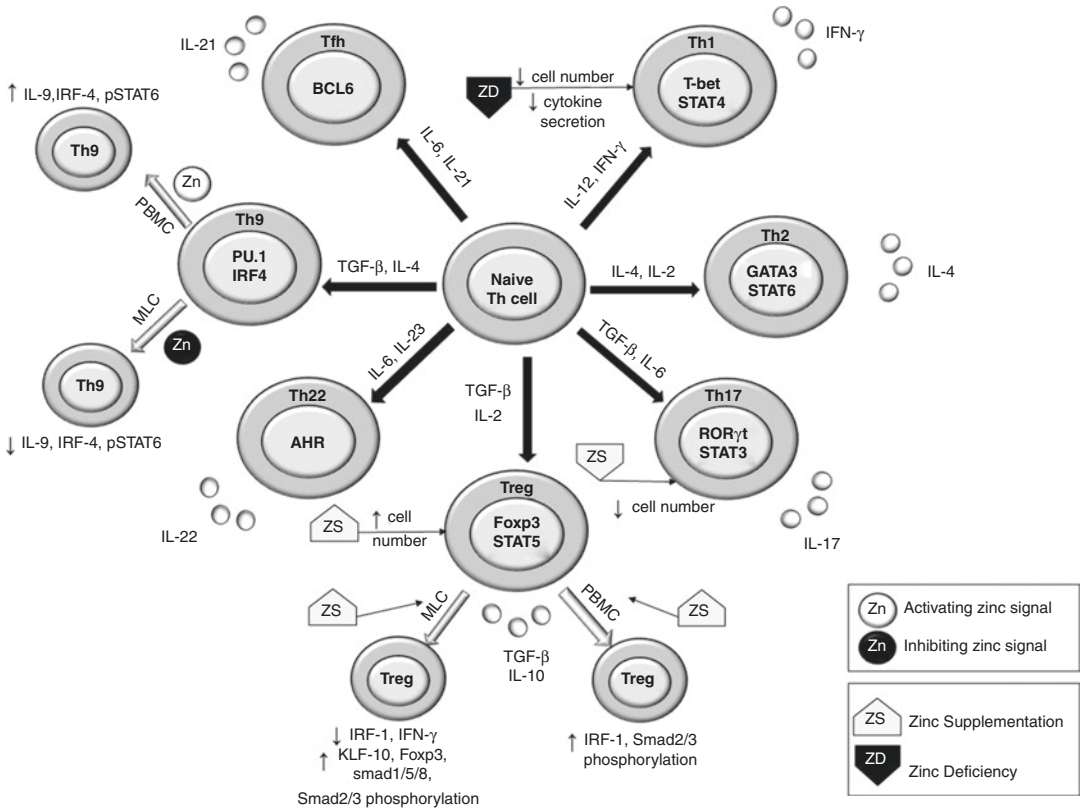

Fig. 8.3 T helper cell subpopulations and how zinc influences different subsets

a decreased ratio of $CD4^+$ to $CD8^+$ cells [133]. However, the development of cytotoxic precursor T cells as well as the function of peripheral CTLs is impaired under ZD conditions [134]. During ZD in mice, CTL killer activity towards allogeneic tumor cells is abrogated [135, 136]. Reduction on proliferation and activation of CTLs during ZD is probably associated with reduced IL-2 production, as IL-2 is an early factor for clonal expansion and differentiation of CTLs [137].

IL-2 stimulation leads to the activation of three different signaling pathways: the STAT5 pathway, the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the extracellular signal-regulated kinases (ERK) 1/2 pathway [138]. When IL-2 binds to its receptor, zinc is released from zincosomes and lysosomes which leads to zinc flux. Zinc regulates IL-2 signaling pathway via blocking MAP kinase phosphatase (MKP) in the ERK 1/2 pathways [137] and phosphatase and tensin homologue (PTEN) which opposes PI3K function in the PI3k/Akt pathway [139]. However, zinc does not seem to affect STAT5 signaling in T cells. Figure 8.4 summarizes the influence of zinc on IL-2 signaling.

Moreover, zinc signals affect T-cell activation by influencing the TCR-activating complex. TCR lacks intrinsic kinase activity and is dependent on Src-family tyrosine kinases for signal transduction, such as lymphocyte protein tyrosine kinase (Lck). CD4 recruits LcK to the TCR. Lck is mainly required for the early phosphorylation events of tyrosines within the ITAM motifs of the T-cell antigen receptor complex and the z-chain-associated protein kinase (ZAP)70 [140, 141]. Zinc facilitates the binding of Lck to CD4 and CD8. Therefore, it stabilizes the signaling complex important for T-cell activation. Additionally, the activity of Lck is influenced by zinc, because zinc modulates the activity of a variety of phosphatases and kinases responsible for regulating Lck activity. Lck plays a pivotal role in the phosphorylation of

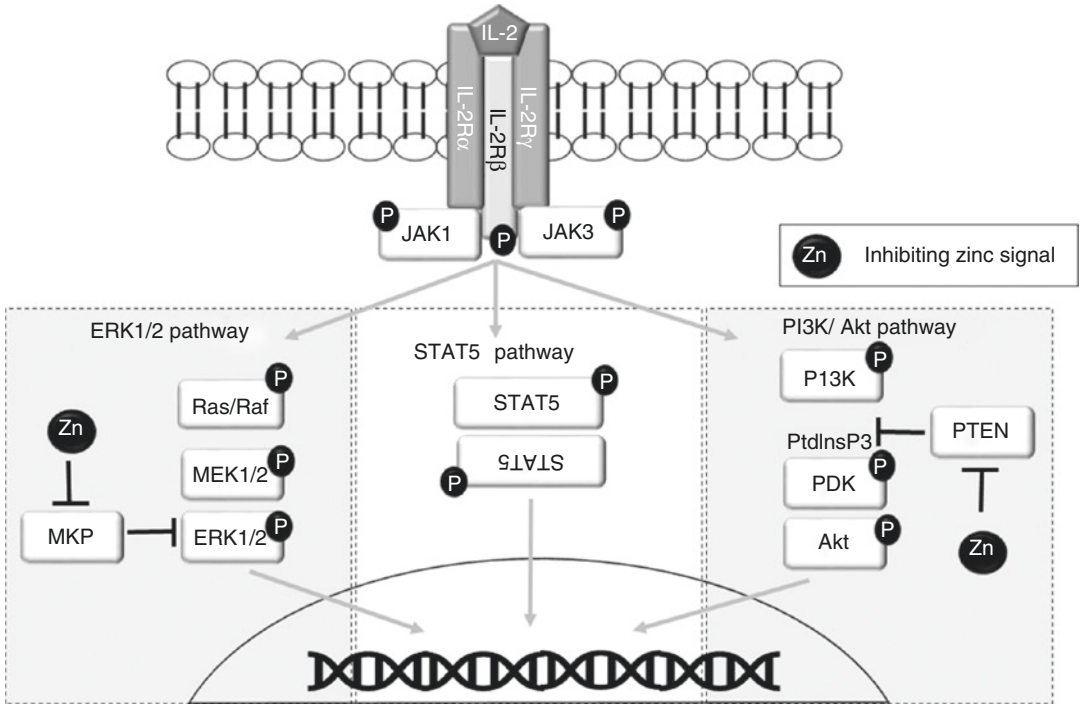

Fig. 8.4 Influence of zinc on IL-2 signaling pathways. (Adapted from [136, 138])

ZAP70, which then further activates signaling molecules such as MAPK. However, abolishing the initial zinc flux affects all downstream signaling events. Increased zinc flux also mediates T-cell activation and Lck activity by decreasing the recruitment of the PTP enzyme SHP-1 to the TCR activation complex.

The initial zinc flux is mediated by several ZIP transporters and occurs within less than a minute. TCR activation induces clustering and activation of ZIP6 transporters in the synaptic region. Silencing *zip6* abrogated this fast zinc signal which demonstrates that the source of zinc is extracellular [141]. Furthermore, zinc signal might also be generated from lysosomal compartments via ZIP8 which is upregulated after TCR activation [142].

Increased cytoplasmic zinc concentration after TCR activation which also inhibits the negative feedback mechanism by suppressing SHP-1 recruitment results in reduced TCR activation threshold allowing it to respond to suboptimal antigen conditions. This may be useful particularly in vaccine optimization as zinc supplementation can induce T-cell proliferation even when stimulated with suboptimal antigen concentrations.

The release of zinc from lysosomal compartments via ZIP8 leads to the reduction of calcineurin (CN) phosphatase activity. This results in activation of cAMP response element-binding protein (CREB) and subsequent expression of IFN-γ. Another well-known target of CN phosphatase is a transcription factor named nuclear factor of activated T cells (NFAT). NFAT is important for IL-2 expression and it is dephosphorylated by CN. When zinc inhibits CN phosphatase, the nuclear translocation of NFAT is prevented. Therefore, the events occurring after TCR activation that lead to increased IFN-γ and reduced IL-2 are regulated by zinc in a concentration-dependent manner [103]. Figure 8.5 summarizes how zinc influences TCR signaling on multiple levels.

Alteration of zinc homeostasis influences all T-cell subsets on different levels. T-cell development, polarization into effector cells, and functional activities are all influenced by zinc.

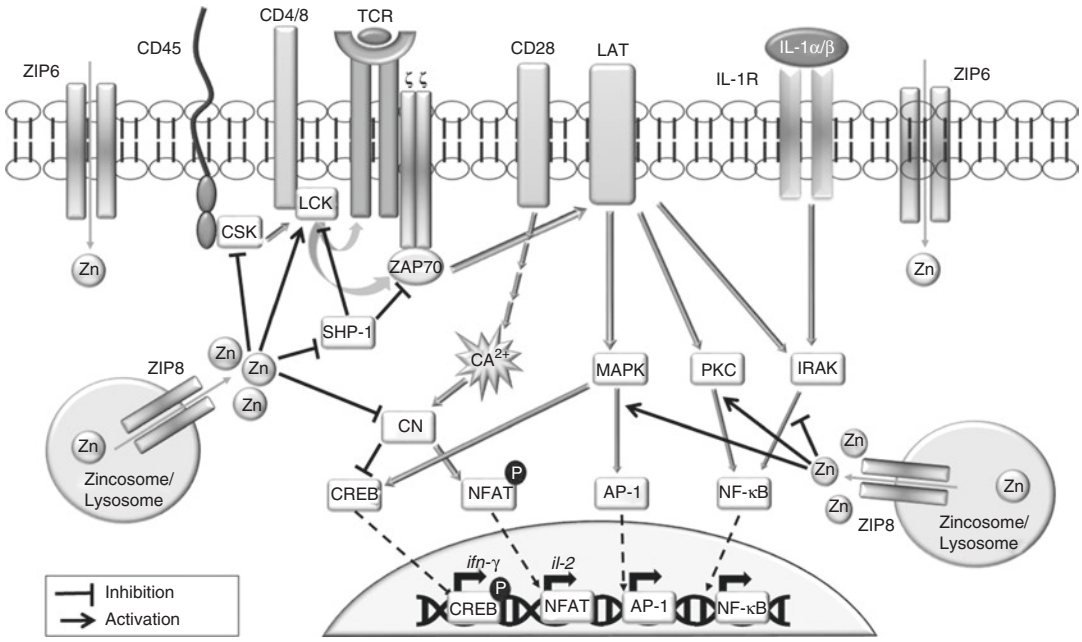

Fig. 8.5 Influence of zinc on TCR signaling pathways. For further explanation see text. (Adapted from [36, 37])

B Cells

B cells make up 5–15% of the human blood lymphocytes. They are key players in the humoral immune response. Their main job is to secrete soluble immunoglobulins that bind specifically to an antigen and subsequently cause neutralization or opsonization. As mentioned previously, once a B cell encounters its correspondent antigen and receives a signal from Th cells, it becomes activated into antibody-secreting plasma cells and undergoes clonal expansion. B cells can also be activated independently from T cells.

B cells are influenced by zinc, but to a lesser extent compared to other immune cells. Nevertheless, ZD influences lymphopoiesis and pre-B cell development mainly through increasing apoptosis which leads to decreased number of naïve B cells. On the other hand, mature B cells are more resistant to apoptosis possibly due to higher expression of antiapoptotic molecule Bcl-2 [143]. Furthermore, the role of zinc in B-cell development was recently investigated. It has been revealed that mice deficient in *Zip10* had a marked reduction in the proliferation of mature B cells and in the production of immunoglobulins in response to BCR signaling. This suggests that ZIP10 plays an important role in regulating BCR signaling and proper B cell functions [144].

Signaling pathways in B cells resemble those in T cells. However, zinc influences the signaling of the B-cell activating factors IL-6 and IL-4 through STAT3 and STAT6, respectively. The phosphorylation of IL-4-induced STAT6 was reduced during ZD, while the phosphorylation of IL-6-induced STAT3 was increased. Since IL-4 promotes the activation of early B cells and the immunoglobulin class switching to IgE, ZD individuals are more susceptible to severe outcomes of parasitic infections. Moreover, IL-6 is required for the activation and final differentiation of B cells into antibody-producing plasma cells. Over production of IL-6 is associated with several autoimmune diseases such as multiple myeloma and rheumatic arthritis. These diseases are correlated with unregulated B-cell growth and production of autoantibodies. They are also linked to reduced serum zinc which support the relationship between ZD and IL-6 overproduction. Zinc supplementation can be used as a therapeutic tool to help reduce levels of IL-6 [145].

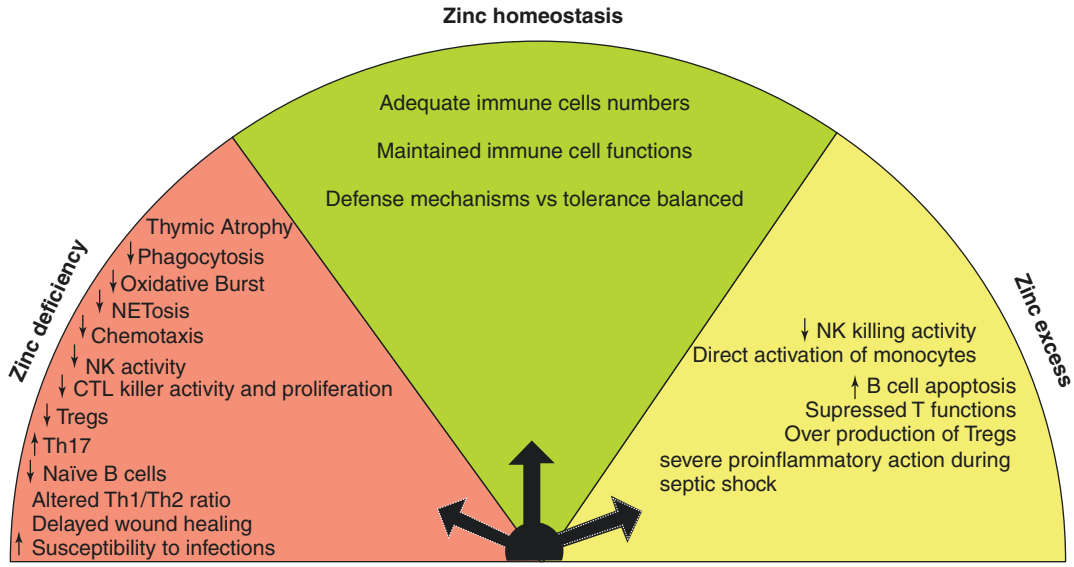

Fig. 8.6 Maintaining zinc homeostasis insures proper functioning of the innate and the adaptive immune system. Zinc deficiency and zinc excess compromise the immune system and lead to a variety of negative consequences that are usually corrected by restoring zinc homeostasis

It is also interesting to investigate the influence of zinc supplementation on vaccine efficiency. Many studies have been carried out with contradictory results. ZD individuals revealed diminished immune response to vaccines [146–148]. However, the optimal level and duration of zinc supplementation should be investigated further. Some studies revealed that excessive amounts of zinc supplementation at the time of vaccination can result in poor outcomes and may inhibit T cells function and reduce B-cell antibody production [149].

Figure 8.6 provides an overview of several outcomes that were observed during ZD or zinc excess conditions and how maintaining proper zinc levels insures adequate immune cell function.

Zinc Deficiency in the Elderly

The correlation between immune functions and zinc status in the elderly population has been repeatedly investigated. About 30% of the elderly population is considered to be ZD. There are several causes of ZD in the elderly. It is typically caused by inadequate zinc dietary intake in the aging population. This is due to, but not limited to, altered diet, inadequate mastication, and changes in intestinal absorption or drug interaction such as diuretics. Some of the clinical manifestations associated with ZD in the elderly are impaired immune response, diarrhea, macular degeneration, decreased resistance to infections, and osteoporosis. Furthermore, there is an evident relationship between age and increased susceptibility to infections, inflammatory and autoimmune diseases, cancers, and other chronic illnesses [150].

As discussed previously, all immune cells are influenced by ZD. In elderly individuals, several key functions of PMNs are impaired, such as chemotaxis, phagocytosis, and oxidative burst [151, 152]. PMNs also display increased susceptibility to apoptosis [153] and reduced SOD which requires zinc for proper functioning [154, 155]. Furthermore, it has been observed that the Th1/Th2 ratio is altered with age, with a shift toward Th2 [156].

In addition to altered T cells functions, B-cell numbers have been shown to decrease with age while nonspecific immunoglobulins and autoantibodies from classes IgG and IgA seem to increase [157]. Elevated secretion of pro-inflammatory cytokines such as IL-6 is also observed in elderly

individuals. Increased IL-6 can be treated with zinc supplementation, which indicates how zinc can significantly reduce inflammatory activity in the elderly [158]. T- and B-cell functions, as described previously, are negatively influenced by ZD. Moreover, the response to vaccination was reduced in elderly individuals with marginal ZD. Some studies show that this response may be enhanced with zinc treatment as demonstrated in the study with tetanus toxoid vaccine [159]. There are also studies indicating that zinc supplementation can significantly reduce the incidence of infections in the elderly [160, 161].

Zinc supplementation or correcting dietary zinc intake can significantly and positively impact the immune system of the aging population and consequently prevent or lower the risk of age-related diseases.

Zinc Deficiency in Infectious Diseases

Infectious diseases are the leading cause of morbidity and mortality among young children, particularly in low-income countries. Diarrhea is a symptom often accompanying an infection and is the second leading cause of death in young children resulting in around 525,000 death every year. Diarrhea that persists for several days can leave the body dehydrated and malnourished [162, 163]. Undernutrition is prevalent in populations suffering from high infection rates. Evaluating the micronutrient status of individuals and the population can help mitigate risk factors and establish preventative measures as well as including those micronutrients in the treatment plans. Zinc deficiency has been linked to many infectious diseases. Many clinical trials have been carried out to explore the influence of zinc supplementation on a range of bacterial, viral, and parasitic diseases. Results vary widely and some are even contradictory. Caution must be taken when drawing conclusions from single studies as several variable factors may influence the generalization of the results. Such factors are dosage and type of zinc supplementation used, duration of supplementation, biomarkers for zinc status, initial zinc status of study participants, and variable health conditions that might influence nutrient absorption [164]. All these factors tend to vary in each study and greatly influence the final conclusions. Table 8.3 provides

Table 8.3 Influence of zinc supplementation on different viral, bacterial, and parasitic diseases

Disease	Number of studies	Daily dosage and treatment period	Effect of zinc supplementation	Zinc supplementation recommendation
Common cold	>12 studies	4.5–23.7 mg	↓ duration of symptoms [165] (zinc administration within 24hrs of illness onset)	Recommended
HIV/AIDS	9	10–50 mg 6 days–6 months	↓ diarrhea [166, 167] ↓ viral load [168] ↑ $CD4^+$ cell count [169, 170] ↑ or delay frequency of opportunistic infections [170] No effect on viral load [167] No effect on $CD4^+/CD8^+$ ratio [171] No effect on birth outcomes of HIV-positive women [172] No effect on response to pneumococcal vaccine [173]	Unclear
Hepatitis C virus	4	10–150 mg 2 months–6 months	↓ α-fetoprotein levels [174] ↓ alanine aminotransferase levels [175] ↓ HCV RNA levels [175] ↓ gastrointestinal disturbances [176] ↓ body weight loss [176] ↓ anemia [176] ↑ response to INF-α therapy [177]	Recommended

(continued)

Table 8.3 (continued)

Disease	Number of studies	Daily dosage and treatment period	Effect of zinc supplementation	Zinc supplementation recommendation
Diarrhea	Numerous	Variable	↓ duration, severity, and incidence of diarrhea [178]	Highly recommended
Respiratory tract infections (RTI)	5	5–20 mg 5 days–12 months	↓ acute lower RTI morbidity [179] ↓ episodes of acute lower RTI [180, 181] ↑ infection-free days [180] ↓ incidence of upper RTI [182] ↑ recovery rate [183]	Recommended
Tuberculosis	1	15 mg 6 months	↑ plasma retinol concentrations Earlier sputum conversion Earlier resolution of x-ray lesion area[184]	Unclear
Lepromatous leprosy	4	200–220 mg 13 weeks–18 months	↓ incidence and severity of erythema nodosum leprosum [185] ↓ dose of clofazimine [186] ↓ steroids requirement [186, 187] ↑ toleration of dapsone [186] ↓ size of granuloma and bacterial load [185, 186, 188] ↑ number of lymphocytes [186]	Recommended
Shigellosis	4	20 mg OR 1.3 mg/Kg 3 times a day 2 weeks–1 month	↑ intestinal mucosal permeability [151] ↑ lymphocyte proliferation in response to phytohemagglutinin and plasma invasion plasmid-encoded antigen-specific IgG titer [189] ↑ shigellacidal antibody titer [190] ↑ $CD20^+$ cells and $CD20^+CD38^+$ cells [190] Faster recovery from acute illness [191] ↑ mean body weight [191] ↓ episodes of diarrhea [191]	Unclear
Malaria	6	10–40 mg 4 days–15 months	↓ prevalence of malaria [192, 193] ↓ number of episodes [192, 194] Delay of first malaria episode [192] ↓ morbidity due to diarrhea [195] No effect [196, 197]	Unclear
Leishmaniasis	1	2.5, 5.0, 10 mg/Kg 45 days	↑ cure rate ↓ erythema ↓ induration size [198]	Unclear

a summary of some of the major infectious diseases and the influence of zinc on the overall clinical picture.

Conclusions

In conclusion, the role of zinc in the immune system is critical. It is required for proper development and functioning of every immune cell. Altered zinc homeostasis, as discussed above, modulates both the innate and adaptive immune response. ZD can have devastating consequences on cellular and systematic levels. Increased susceptibility to infection and autoimmune diseases are among the common issues associated with ZD. Fortunately, observed negative outcomes of ZD can be reversed with proper zinc supplementation and impaired immune functions can be restored as seen by many clini-

cal trials. Zinc is an excellent anti-inflammatory immunomodulator. As more studies reveal its role at a molecular level, the better the understanding of how zinc can be exploited as a therapeutic agent.

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Selenium and Immunity

9

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Key Points

- Selenium (Se), like vitamin A, C, and E and zinc, is an exogenous antioxidant, capable of scavenging free radicals, originating either from the cell itself or from the external environment.
- Low selenium intake is associated with increased levels of peroxides and increased disease progression.
- Adequate Se intake enhances both cell-mediated and humoral response. However, Se intake in association with

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other micronutrients significantly improves disease outcomes.
- Se intake through Se-rich food such as Brazil nuts, sunflower seeds, lean pork, seafood etc. is ideal. In regions with low soil Se, it can be supplemented according to the age and health condition.
- Supplementation of Se can prevent the development of both bacterial (*Mycobacterium tuberculosis*) and viral (Keshan disease, human immune deficiency virus) infections. Meanwhile, excess Se supplementation could result in decreased immunity and increased oxidative stress.

Introduction

Immunity is the ability of the body to resist harmful microorganisms such as viruses, bacteria, and foreign bodies and to prevent them from invading its environment. Immunity can be influenced by factors such as hormones [1, 2], sleep [3], aging [4], and nutrition. The relationship between nutrition and immunity is well established in previous and recent studies [5–8]. The nutritional status of an individual is influenced by the nutrient intake with food or in supplements. Inadequate intake of these nutrients could lead to nutritional deficiencies. About two billion people worldwide are reported to be deficient in one or more micronutrients [9, 10]. Selenium is an essential micronutrient with known antioxidant properties. Antioxidants are molecules produced either in situ (GSH, glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; catalase; TrxRs, thioredoxin reductases) or externally supplied to the body through the diet or with supplements (vitamins A, C, E, selenium, and zinc). Both endogenous and exogenous antioxidants are capable of scavenging free radicals – originating either from the cell itself or from the external environment (cigarette smoke, radiation, and pollution) – by donating an electron to a rampaging free radical, neutralizing it, and reducing its ability to damage the system [9, 11].

Sources of Selenium

Selenium is naturally present in the food we eat. It is abundant in Brazil nuts, seafood such as shrimps and oily fish, meat, and meat products. The selenium content in foods varies by geographical area. Soils contain inorganic selenium that are converted into organic selenium in plants. Soils with low selenium concentrations and low dietary intakes are the most common causes of selenium deficiency [12]. Selenium is also available as supplements in the form of capsules, tablets, powders, syrups, drinks, and energy bars [13]. However, it is important to note that besides its advantages, selenium is required by the body in limited amount; thus excessive intake could lead to toxicity. Meanwhile abusive intake of dietary supplements may be accompanied by severe side effects. For example, some may interact with certain drugs, thereby provoking diverse harmful effects. It is therefore advisable to take supplements according to a medical practitioner's recommendation.

Recommended Daily Allowance

According to the National Institute of Health (NIH), selenium intake and concentration in the USA and Canada vary from region to region due to differences in selenium content of the soil and consequently local foods. The European Union provides a population recommended intake value (PRI-value) according to scientific tests and activation of GPx.

Selenoproteins

Selenium is a structural component of the amino acid selenocysteine that is incorporated into 25 selenoproteins. These proteins take part in a variety of physiological functions in the mammalian systems. Genetic defects in selenocysteine resulting in selenoprotein insufficiency have been correlated with health issues. One of the most important selenoproteins is glutathione peroxidase (GPx). GPx belongs to an enzyme family with peroxidase activity, whose key biological role is to

prevent the organism from oxidative harm. GPx can effectively reduce H2O2 and lipid peroxides to water and lipid alcohols as well as oxidize glutathione to glutathione disulfide. Glutathione is important in the regulation of the intracellular redox state of vascular cells by reduction of equivalents for numerous biochemical pathways. When GPx activity and/or glutathione levels are not sufficient, hydrogen peroxide and lipid peroxides turn into OH radicals and lipid peroxyl radicals by transition metals (Fe2+). The GPx/glutathione method is thought to be the main advocacy in low-level oxidative stress. Four isoforms of GPx have been characterized. GPx-1 (cellular GPx) is ubiquitous and deals with the reduction of H2O2 and fatty acid peroxides. It has been inversely correlated with a higher risk of cardiovascular events. GPx2 (gastrointestinal GPx) is restricted to a small group of gastrointestinal epithelial cells. GPx-3 (extracellular GPx) is the only member of the GPx family that is present in the extracellular space. It is considered as the major extracellular antioxidant enzyme in mammals [14, 15]. Finally, esterified lipids are reduced by membrane-bound GPx-4 (phospholipids hydroperoxide GPx). GPx4 is expressed in epithelial cells and the lamina propria of the intestine.

Particularly, cellular and plasma glutathione peroxidase are used as a measure of selenium status. Thioredoxin reductase (TR) is a recently recognized selenocysteine enzyme which catalyzes the NADPH (nicotinamide adenine dinucleotide phosphate)-dependent reduction of thioredoxin (Trx). About 60% of selenium in plasma is found in selenoprotein P. Selenoprotein P containing 10 selenium atoms in the form of selenocysteine per molecule serves as a transport protein for selenium. Selenoprotein P is also detected in a variety of tissues. This indicates that selenium is distributed throughout the body whereby it can cooperate with multiple biological functions. A second major class of selenoproteins are the iodothyronine deiodinase enzymes which catalyze the 5′5-mono-deiodination of the prohormone thyroxine (T4) to the active thyroid hormone 3,3′5-triiodothyronine (T3). Sperm capsule selenoprotein is found in the mid-piece portion of spermatozoa. Thereby it might help with stabilization of the integrity of the sperm flagella. Selenium intake would influence tissue concentrations of selenoprotein W, which is thought to be essential for muscle metabolism. Additionally, selenium has been shown to protect against tumorigenesis, improve male fertility, reduce cardiovascular disease mortality, and control the inflammatory response in asthma [16]. Of particular interest to the present chapter is that dietary selenium plays a crucial role in maintaining a healthy immune system.

Selenium and the Immune System

A majority of micronutrients in the diet, including selenium, play a fundamental role in maintaining an “optimal” immune response. As a component of selenoproteins, selenium is required for the effective functions of neutrophils, macrophages, NK cells, and T lymphocytes. High selenium intake may help relieve oxidative stress and inflammation and is linked to a decreased risk of cancer. Selenium acts to improve virulence of HIV and thereby inhibiting its progression to AIDS. It is required for sperm mobility and may decrease the risk of miscarriage. Selenium deficiency has been correlated with higher risk of adverse mood states and cardiovascular diseases.

Selenium and Age-Linked Decline in Immune Cell Function

Dietary supplementation of selenium showed a significant increase in the ability of the spleen lymphocytes from aged animals to experience blastogenesis. This was justified by an increased level of nuclear interpolation of 3H-thymidine after mitogen stimulation. In addition, the in vivo study revealed that selenium-supplemented aged animals could benefit from greater numbers of cytotoxic lymphocytes than those from untreated aged animals. Thereby, selenium supplementation could strengthen the capacity of the immune system to destroy tumor cells. The increase in the number of cytotoxic effector cells within the activated T-cell populations was possibly the result of an improved clonal propagation of cytotoxic

precursor cells, followed by the separation of greater numbers of cytotoxic effector cells. However, this effect occurred to cytotoxic function of T cells while maintaining the quantity of IL-2 production of the cells [17].

Selenium Deficiency and Disease

Selenium plays a vital role in cell-mediated immunity. It is, thus, expected that selenium deficiency would result in an increased susceptibility to viral and bacterial infections and associated mortality [18].

Viral Infections

Viral infections cause an increase in the generation of reactive oxygen species (ROS) and thus downregulate the biosynthesis of antioxidant enzymes in the infected cell [19]. Viral infections often correlate with deficiencies in macronutrients and micronutrients, favorably selenium. Selenium deficiency is common among patients infected with viruses. For example, coxsackievirus is an enterovirus that causes Keshan disease characterized by gastrointestinal distress, full-fledged pericarditis, and myocarditis (coxsackievirus-induced cardiomyopathy). Patients infected with this virus are very likely to have severe selenium deficiency with blood selenium concentrations below 20 μg/L (0.25 μM) [20, 21]. Evidence suggests that selenium supplementation could completely prevent the development of Keshan disease by enhancing viral immunity and by preventing genetic adaptations in the viral genomic RNA, together resulting in reduced virulence and cardiac pathology. In animal studies, heart damage was observed upon infection with a non-cardiovirulent strain of coxsackievirus B (CVB3/0), only in selenium-deficient mice, whereas mice fed selenium-adequate diets (0.2 ppm selenium as selenite) showed no evidence of heart damage. This was reflected in a higher viral load in the heart of the selenium-deficient mice as well as in a lower antigen-specific T-cell response compared to that of the selenium-adequate littermates. Mice carrying an aberrant selenoenzyme ($GPx1^{-/-}$) gene developed myocarditis after infection with the benign CVB3/0 strain. A majority of the nucleotide interactions in viruses isolated from infected $GPx1^{-/-}$ mice was comparable to those found in mutated viruses from selenium-deficient mice. This suggests that selenium protects from ROS-induced mutations of the viral RNA genome through the action of GPx1 [10].

The human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS), is one of the important causes of mortality worldwide and especially in sub-Saharan Africa. HIV infection is an RNA viral infection capable of causing progressive failure of the immune system if untreated. Varieties of efforts are ongoing to reduce the burden of HIV infection. Among which, improving antiretroviral therapy (ART) and promoting nutritional interventions are of important value. ART is not a definitive cure for HIV/AIDS but is able to suppress the viral replication and improve CD4 counts [22]. However, ART availability is still limited in most sub-Saharan African settings, and its use is associated with side effects such as changes in the distribution of body fat, insulin resistance, and fatigue. As a result, patient adherence to ART is low, leading to drug resistance [23].

A healthy nutrition and a healthy immune system are fundamentally linked. In particular, micronutrients play a vital role in the care and management of patients with HIV. Micronutrient deficiency including selenium deficiency commonly occurs in patients with HIV. In addition, patients with AIDS had lower selenium concentrations and GPx activity compared to patients with asymptomatic HIV and healthy population. Low levels of glutathione and GPx activity in CD4+ cells increase the levels of peroxides, which, in turn, stimulate apoptosis, thereby destroying the HIV-infected cells. This implies that serum selenium concentrations correlate positively with disease progression and risk of mortality. It is also known that HIV-1 expression is regulated by nuclear factor kappa B (NF- κB) through the redox-controlled signal transduction pathway. Selenium supplementation can increase GPx activity in dormant infected T cells, thus protecting them against hydrogen peroxide and decreasing NF-kB activation in selenium supplemented cells [19].

Meanwhile, marginal selenium deficiency in patients with HIV is associated with increased shedding of HIV-infected cells in the genital tract [20]. Although results are still inconsistent, evidence suggests that a high selenium intake along with other micronutrients such as vitamin mixture (B vitamins, vitamins C and E) could significantly delay the destruction of CD4+ cells and onset of AIDS and risk of comorbidity. However, how effective selenium supplementation depends on the degree of deficiency in these patients. In addition, when compared with supplementation of selenium alone, selenium supplementation in combination with other micronutrients was more effective in improving the selenium status of patients. Other factors affecting the effectiveness of selenium supplementation include the form in which selenium is being supplemented (selenite or selenomethionine) and stage of HIV infection.

Bacterial Infections

Little evidence exists on whether selenium helps the body against bacterial infections. Nevertheless, the provision of selenium compounds in combination with multivitamins has gone a long way to improve nutritional status and weight gain in patients suffering from *Mycobacterium tuberculosis* [24]. Studies suggest that nutritional challenges in patients with tuberculosis, including macronutrient and micronutrient deficiencies, malabsorption, and increased metabolic demands, exacerbate the severity of infection and slow down treatment process [25]. In addition, animal studies provide evidence that the immune response is influenced by selenium status following bacterial infection. Both the innate and humoral immune responses were impaired in selenium-deficient sheep affected by foot rot. Selenium supplementation could restore immune function though it was not able to prevent foot rot [26].

Cancer

Meta-analysis has strengthened the evidence linking high selenium to a decreased risk of cancers. The effect of selenium tumorigenesis is mediated by its influence on cell cycles, apoptosis, DNA damage and repair, cell adhesion and migration, angiogenesis, and immunity. Clinical studies are warranted to determine the dosage and chemical form of selenium supplement to be included in cancer therapy [27].

Meanwhile selenium dietary intake is suboptimal in many regions of the world. Given the antitumorigenic properties of selenium, it is expected that the risk of cancer and associated morbidity and mortality would significantly decrease if selenium supplementation were included in the public health plans. Additionally, selenium can sequestrate other elements available in foods, water, and the workplace. The sequestration of these elements by selenium is an effective detoxification mechanism. Animal studies note the connection of the chronic exposure to the aforementioned heavy metals and cancer. For instance, cadmium has been associated with an increased risk of prostate cancer, cadmium, chromium, and zinc with breast cancer, and cadmium, arsenic, chromium, antimony, cobalt, and lanthanum with bronchial cancer. All these elements are in mutual connection with selenium. Thus, selenium remains a potential candidate biomarker for cancer [28].

Further, TrxR, a recently recognized selenocysteine and one of the well-documented selenoproteins in cancer enzyme plays an important role in the prevention, treatment, and diagnosis of cancer. TrxR produced by pre-neoplastic and tumor cells can promote tumor progression and development of the resistant phenotype of cancer.

Regulation of Inflammation by Selenium on Eicosanoid

Abnormal inflammation is a contributing factor to numerous leading causes of morbidity and mortality, related to atherosclerosis, cancer, and diabetes. Selenium is an important nutrient in the mammalian diet that has several anti-inflammatory attributes. Adequate intake displays protective effects against inflammatory conditions. Recently, selenium has been demonstrated to alter the expression

of eicosanoids that orchestrate the initiation, maintenance, and resolution of inflammation. An improved understanding of the mechanisms implicated in selenium-mediated regulation of host inflammatory responses can help to design dietary plans that take optimal benefit [29].

Selenium and Brain Function

Selenium appears to contribute to the pathophysiology of neurological conditions, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and epilepsy. Selenium plays a role in various functions of the central nervous system, such as motor performance, coordination, memory, and cognition. Selenoproteins are of particular importance in improving the functioning of GABAergic (GABA, γ-aminobutyric acid) neurons of the cerebral cortex and hippocampus. In addition, selenium has been shown to influence dopaminergic function and acetylcholine neurotransmission. The character of selenium and selenoproteins in neurotransmission might not only be confined to their antioxidant attributes but also to inflammation, protein phosphorylation and ion channels, modification of calcium homeostasis, and brain cholesterol metabolism [30].

Selenium and Senescence

Senescence is a biological phenomenon that leads to a permanent arrest in the cell cycle, resulting in the termination of physiological functions such as embryogenesis and tissue regeneration. Cellular senescence appears to be caused by DNA injury, dysfunctional telomeres, chromosomal conformation changes, and mutagenic stimuli. Evidence indicates that selenium can postpone senescence and related disorders including cancer, neurodegeneration, and metabolic syndromes [31].

Selenium Toxicity

As low levels of selenium impair the proper functioning of the immune system, its excess can have detrimental or even fatal effects. The bioavailability and toxicity of selenium vary depending on its chemical form. Commonly, organic forms of selenium are more bioavailable and less toxic than inorganic forms. Selenium toxicity is not common in humans, but excess intake could occur due to miscalculated supplement formulation, intentional poisoning, or unintended overdose. Results from a recent animal study showed that excess selenium supplementation could suppress the immune responses through aggravating oxidative damage along with a series of clinical pathology changes [32].

Conclusions

An important strategy to reduce selenium deficiency worldwide and in sub-Saharan Africa specifically is to increase the intake of selenium-rich food. However, this strategy can only be effective in areas where local foods are rich in selenium. According to researchers, such a strategy will hardly be successful in areas where populations have maize as staple food or only source of selenium. Maize has less than 7 μg selenium/100 g (normal range) [33]. In such areas, selenium supplementation in recommended doses according to age group and health condition is vital. Further, supplementation of selenium of about 200 μg/day in combination with adequate nutrition for people suffering from viral infections will strengthen the immune system and improve treatment efficacy and quality of life of these patients. Meanwhile, it is necessary to educate general populations, especially people with a compromised immune system on the use of selenium-containing crop fertilizer and sources on selenium in local foods.

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Gut Microbiome and Immunity

10

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Key Points

- The gut serve the largest population of microflora which can boost both innate and adaptive immunity.
- Gut microbes extend both a suppressive and a stimulatory effect on the immune system, which can extend beyond the gut to affect several vital organs.
- Dysbiosis of the gut microbiota accompanies or even precedes the development of immune-mediated disorders.
- Dietary factors serve as a promising target for manipulating the gut microbiota to boost the immune system at every stage of life.
- Gut microbiota are a potential target for a broad range of health problems including allergies, infections, autoimmune disorders, neuropsychiatric disorders, cardiometabolic disorders, and cancer.

Introduction

Intestinal epithelial cells (IECs) with an area coverage of 100 m^2 provide the most luxury mucosal barrier that protects the host from undesired antigens [1]. In addition, commensal microflora is present at all body surfaces which are lined with epithelial tissues, as are constitutively exposed to the external environment [2]. In particular, the gut include the largest population of microflora including 10^{14} commensal bacteria [3]. This crucial ecosystem defense against invading pathogens. However, it can become overwhelmed and lead to allergy, autoimmunity and inflammatory diseases, notably inflammatory bowel disease (IBD) and gastrointestinal cancer [1, 4]. In this manner, the gut microbiome plays an essential role in the regulation of the balance between immune tolerance and inflammation [1].

Given the role of the gut microbiome in shaping the total immunity, it would be necessary to unravel factors affecting the gut microbiome. Dietary, as well as environmental antigens, are a key to determine the composition of the gut microbiota. On the one hand, the gut microbiome composition by cooperating with nutrient absorption would directly affect the nutritional value of the food eaten. In addition, pathways which absorbed nutrient components and recognized pathogens have been suggested as the basis for the close relationship between the gut microbiome and immunity. In light of this evidence, a relatively higher rate of autoimmune and inflammatory disorders is not surprising in developed countries, where certain dietary behaviors make people more susceptible to immune-metabolic disorders, especially obesity [5]. Overall, nutrition and dietary factors that would be influenced by culture and socioeconomic status seem most amenable and, of course, appropriate to optimization in the context of immunity in the gut. As a result, our nutritional status reflects the status of both the gut microbiome and the immune system [6].

The present chapter will address first how bacterial microbiota compositions can characterize many aspects of the immune system and then how intake of live bacteria (probiotics) through manipulation of the gut microbiota might affect this characterization.

Microbiome and Immunity

As discussed in the Introduction chapter, there are two main arms of immune responses, innate and adaptive. Adaptive immunity, in turn, can generate cell-mediated and antibody-mediated (humoral) immune responses. The most important component of humoral immunity is B cells, which upon activation (usually by T_H cells), have the ability to recognize unprocessed extracellular antigens and secrete antigen-specific antibodies. Humoral immune responses mainly engage B-cell receptors (BCRs) and immunoglobulin alpha (Igα), Igβ, cluster of differentiation 40 (CD40), CD21, and Fc receptors provide accessory signals. T cells which are a key player in cell-mediated immunity do act on intracellular antigens processed through the major histocompatibility complex (MHC) molecules. Cell-mediated immune responses mainly involve T-cell receptors (TCRs) and CD2, CD3, CD4, CD8, CD28, and integrins act as accessory receptors. Cytotoxic T (T_C) and helper T (T_H) cells critically participate in cell-mediated immunity. Cells infected with intracellular microorganisms are transported to the cell surface with the help of MHC proteins. Then, they are presented to T_C cells to direct cell death program. On the other site, T_H cells induce the activation of macrophages and B cells [7].

Accumulations of commensal bacteria occur in the gut-associated lymphoid tissues (GALTs) that rest throughout the gut [1]. Studies of mice reveal that conventional colonization of the intestine would influence the expression of genes of which about half are involved in the immune response pathways, especially innate and adaptive T-cell immune responses in the terminal ileum [8]. Consequently, absence of such a microbiota can seriously hamper the intestinal immunologic development that cellular defects and molecular immune deficiencies might occur [9]. In particular, immunologically important intestinal sites which include Peyer's patches (PPs), lamina propria (LP), germinal centers (GCs), and isolated lymphoid follicles become less cellular or fewer in plasma cell numbers in germ-free (GF) mice. Plasma cells are responsible for providing adequate amounts of antibodies, which play an important role in the activation of humoral immunity. Cellular defects affect the intestinal epithelial lymphocytes, LP lymphocytes, and mesenteric lymph nodes (MLNs) whereby, respectively, numbers of $CD8^+$ T cells, $CD4^+$ T cells, and $CD4^+CD25^+$ T cells will become lower. Restricted repertoire of $CD8^+$ T cells which are also known as T_C cells has the potential to impair defense against intracellular pathogens such as viruses, some bacteria, and some protozoa [10] as well as the immune surveillance against tumors. Mice lacking $CD4^+$ T cells reveal impaired signaling to T_H 17 responses [11], which are essential to the defense against extracellular pathogens and also the regulation of immune tolerance [12]. Failure in the development of $CD4^+CD25^+$ T-cell lineage decreases the expression of the transcription factor forkhead box P3 (Foxp3) and therefore suppressive capacity of $Foxp3^+$ regulatory T cells (T_{regs}). Thereby, the intestine will become inflamed [13]. Immune deficiencies can occur in molecules such as angiogenin-4, RegIIIγ, secretory immunoglobulin (Ig) A, adenosine triphosphate (ATP), MHC class III, toll-like receptor (TLR) 9, and interleukin (IL)-25 [9].

Immunomodulatory Microorganisms

As the name imply, T_{reg} cells play a role in the regulation of immune homeostasis so to not overwhelm inflammatory responses. These cells are concentrated in different clusters arising from two origins: thymus (tT_{reg}) and peripheral tissues (iT_{reg}). Upon activation, tumor growth factor beta (TGF-β) induces the expression of Foxp3 in CD4+ T cells. $Foxp3^+$ T_{regs} are more potent than $Foxp3^-$ T_{regs} as immunosuppressive cells.

An increased distribution of $Foxp3^+$ T_{regs} is accompanied by increased expression of genes

encoding proteins that interfere with inflammatory processes, while aiding with tumor surveillance and immune tolerance, such as IL-10, tumor growth factor beta (TGF-β), inducible costimulator (ICOS), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and Epstein–Barr virus-induced 3 (Ebi3). The densest accumulation of $Foxp3^+$ T_{regs} is by far found in LP of the colon and to a lesser extent of the small intestine. Study of mice has shown that the intestinal distribution of $Foxp3^+$ T_{regs} predominantly occurs after weaning.

Cyclophilin seven suppressor (CNS) 1 serves as an intronic enhancer facilitating expression of transcription factors, such as FAT, Smad3, RAR/RXR, and Foxp3, which are, in particular, involved in the initiation of iT_{reg} cells and $Foxp3^+$ T_{reg} cells [14, 15]. Study has shown that $CD4^+$ T cells lacking CNS1 are still able to supply normal numbers of tT_{reg} cells that are efficient to mediate immunological tolerance directing the body away from autoimmune diseases [16]. However, CNS1 deficiency by selective impairment of iTreg cell generation shifted the cytokine balance toward T_H2 responses, as reflected in increased expression of IL-4, IL-5, and IL-13 in $CD4^+$ cells resident in MLNs, LP, and PPs. Also, the production of IgA and IgE was increased in CNS1-deficient mice, consistent with increasing GC B cells. Such altered immune responses associated with weight loss and allergic intestinal inflammation could be ameliorated by B-cell depletion. Of interest to the present Chapter is that CNS1 deficiency can alter gut microbiota characterized by relatively higher contents of bacteria belonging to phylum *Saccharibacteria* and genus *Bacteroidetes alistipes* and lower proportion of *Firmicutes* to *Bacteroidetes*.

Clostridium strains are anaerobic spore-forming gram-positive rod belonging to the phylum *Firmicutes*. Study identified *Clostridium* species belonging to clusters IV and XIVa as the most likely gut microorganisms to contribute to the generation of colonic T_{regs} [17]. More precisely, *clostridium* can activate IECs to provide a myeloid differentiation primary response 88 (Myd88)-independent signal for the expression of T_{regs}-inducing molecules such as TGF-β and indoleamine 2,3-dioxygenase (IDO). Of note, colonic $Foxp3^+$ Tregs induced by *Clostridium* are able and promote the activation of T_H17 responses in the colon. The expression of IL-10 to which a myriad of immunosuppressive effects has been attributed occurs in a substantial portion of colonic $Foxp3^+$ Tregs, so-called $Venus^+$ $Foxp3^+$ cells. $Venus^+$ $Foxp3^+$ cells are further able to express CTLA-4 and therefore would exert more immunosuppressive effects. Interestingly, this population of cells continues its increase in peripheral tissues such as the liver, lung, and spleen three weeks after *Clostridium* colonization. This indicates that immune-suppressive effects of Clostridium are not local, but go beyond the intestine.

Bacteroides fragilis (*B. fragilis*) is an anaerobic gram-negative rod commensal to the colon. *B. fragilis* produces polysaccharide A (PSA) that through a TLR2-dependent mechanism can promote differentiation of $CD4^+$ T cells into $Foxp3^+$ Treg cells in the MLNs [18]. Treatment with the combination of *B. fragilis* and *Burkholderia cepacia* could ameliorate colitis induced by CTLA-4 inhibition in mice treated with certain antibiotics (ACS, ampicillin + colistin + streptomycin) [19].

Faecalibacterium prausnitzii (F. prausnitzii) is an anaerobic gram-positive rod residing in the human gut microbiome. Relatively low ileal content of *F. prausnitzii* in patients with Crohn's disease (CD) with endoscopic relapse compared to patients still in remission at six months after surgery proposed anti-inflammatory properties of these bacteria thus allowing to keep patients disease free [20]. Supporting this, *F. prausnitzii* led to a strong increase in concentrations of anti-inflammatory cytokine IL-10, while slightly inducing the expression of pro-inflammatory cytokines IL-12 and interferon gamma (IFN-γ) by peripheral blood mononuclear cells (PBMCs). Further, *F. prausnitzii* diminished IL-8 production and nuclear factor kappa B (NF-κB) activation induced by IL-1β in Caco-2 cells, a model of the epithelial colorectal adenocarcinoma.

As with any mechanism of activation, the aforementioned bacteria can provide effective protection against inflammation.

Inflammatory Microorganisms

Following colonization of gnotobiotic mice by a whole mouse microbiota, pro-inflammatory T_H1, T_H17, and T_{reg} cell responses would predominate in terminal ileum. With this evidence, Gaboriau-Routhiau and colleagues [8] pursued the relative potential of individual bacteria to elicit such response. They found that segmented filamentous bacteria (SFB) is necessary for inducing intestinal T-cell responses and its functions are mediated mainly via TLR signaling and promoting T_H1 responses.

Non-obese diabetic (NOD) mice are used to study type 1 diabetes (T1D). NOD mice lacking adaptor protein MyD88 are resistant to diabetes [21]. Looking at this specific evidence, MyD88-dependent TLR signaling appears to play a crucial role in diabetes. However, when combined with evidence of development of T1D in GF-NOD mice deficient in MyD88, but not in SPF-NOD mice deficient in MyD88, and more interestingly, an attenuated course of T1D after colonization by defined species, it is concluded that (a) microbiota is protective against TID and (b) this role does not depend on MyD88-dependent signaling. Finally, the authors of the study investigated changes in microbiota composition in SPF-NOD mice deficient in MyD88, which showed increased content of bacteria belonging to genera *Lactobacillaceae* and *Bacteroidetes*.

TLR5 that is expressed in the gut mucosa has the ability to recognize bacterial flagella and trigger innate immune responses against bacterial infections. Mice lacking TLR5 display severe inflammation of the intestine, increased body mass and adiposity, hyperphagia, impaired glucose homeostasis, and higher measures of triglycerides, cholesterol and blood pressure, and elevated production of pro-inflammatory cytokines such as IL-1β and IFN-γ [22]. Such a prominent picture of metabolic syndrome was not evident in MyD88-deficient mice, emphasizing the crucial role TLR45 plays against metabolic syndrome. Altered species composition of gut microbiota in addition to the ability of T5KO microbiota to develop metabolic syndrome in GF-wild type (WT) mice suggest that TLR5 deficiency involvement in metabolic syndrome may be mediated by influencing gut microbiota composition. This difference across studies can be explained by the type of immune responses used in model of colitis [23]. Accordingly, engagement of TLR signaling pathways is particularly observed when innate immune responses are largely responsible for the development of intestinal inflammation and colorectal cancer [24, 25], while adaptive immune responses mainly involve non-TLR-dependent signals to cause colitis and cancer [26].

An attenuated course of rheumatoid arthritis (RA) along with reduced measures of autoantibodies and IL-17-producing T cells in GF animals compared to conventionally colonized animals proposed that the gut microbiota might play role in the progression of disease [27]. Finally, the study suggested SFB as the subversive gut microorganism, because monocolonization of GF mice with these bacteria led to the development of RA.

Similarly, the observation that conventional colonization confers increased susceptibility to multiple sclerosis (MS), an autoimmune disease of the central nervous system (CNS), posed the possibility that microbiota microorganisms play role in the induction of MS. Studies of experimental autoimmune encephalitis (EAE), an animal model of MS, in mice have revealed that SFB contribute to the development of EAE by inducing T_H1 and T_H17 cell responses and double-positive T cells and increasing the expression of pro-inflammatory cytokines IL-17A and IFN-γ in the spinal cord, which all have been associated with autoimmune disease [28].

Activation-induced cytidine deaminase (AID) deficiency results in an autosomal recessive form of hyper IgM syndrome, characterized by normal or high IgM levels and remarkably low levels of IgG, IgA, and IgE [29]. AID deficiency would alter the composition of gut microbiota [30]. The upper and lower part of the small intestine are dominated by SFB and uncultured anaerobic bacteria (SFB and bacteria belonging to *Clostridium* species) in mice lacking AID, and by aerobic bacteria belonging to *Lactobacillus* and anaerobic bacteria (SFB) in age-matched WT mice.

Therefore, AID deficiency converts intestinal microbiota into a more anaerobic state. Such an effect is also observed with aging. Interestingly, transfer of bone marrow (BM) cells from WT mice to mice lacking RAG2, which reveal relatively high content of SFB, resulted in a reduced abundance of anaerobic bacteria. In contrast, transfer of BM cells from AID-deficient mice led to sustained expansion of anaerobic bacteria in RAG2-deficient mice. Consistently, anastomosis with gut normally expressing IgA effectively recovered AID-deficient mice from isolated lymphoid follicle hyperplasia, as well as caused a decrease in the lymph node size and spleen dimensions.

Probiotics and Immunity

Among the mechanisms of action of probiotics, the immune-modulatory signaling impacts the epithelium of LP [31]. Moreover, soluble factors released by probiotics can trigger signaling cascades that target epithelial cell adhesion [32]. However, more research is needed to detect which kinds of probiotics can be very powerful in influencing epithelial cells and forming a biofilm on their surfaces [33].

Inactivated Probiotics

The scientific literature believed that the balance of T_H cell is crucial for cell [34]. Some research has demonstrated the inactivated probiotics can ameliorate IgE production, shift T_H2 response into a T_H1 or T_H0/T_{reg}, and manage allergy response [34, 35]. Furthermore, even the inactive forms of probiotics are able to treat IBD [36]. In recent years, the beneficial effects of probiotics predominantly were shown to redistribute immune responses toward a T_{reg}/T_H0 type profile and thereby minimizing the T_H2 responses [34]. The most marked component of inactivated probiotic are cell wall fractions which have anti-inflammatory effects on dendritic cells [37].

Several other researches have reported that probiotics up-regulate IL-10 by dendritic cells while decreasing IFN-γ production by T cells in a dose-dependent manner [34].

The in vitro study suggested that treatment of human PBMCs with inactivated probiotics (B. *Coagulans* GBI-30, 6086 cells) would activate anti-inflammatory cytokines and chemokines, especially in post-injury and post-inflammation conditions [38]. In addition, high levels of granulocyte-colony stimulating factor (G-CSF) were evident. These data support the idea that lipoteichoic acid of the cell wall is not affected by the inactivation process of probiotics [38]. Moreover, the synergic effects of different probiotic strains could alter and enhance immune responses in comparison with a single strain [39].

The results from the randomized clinical trial on healthy elderly subjects treated with heated-killed *Lactobacillus Gasseri* TMC0356 (TMC0356) showed that an inactivated probiotic could increase the $CD8^+$ T-cell count while declining $CD28^+$ T cell count [40]. Consequently, heated-kill probiotics can contribute to innate immune responses against infection [40].

Furthermore, both experimentally and clinically, it has been shown that gut microbiome plays a vital role in cellular responses, favorably immunity and metabolism [41]. An altered microbiome can affect cancer immunity [41]. This valuable result would change the future of cancer treatment by the combination of probiotics and anti-neoplastic agents [41]. In addition, probiotics have the ability to increase the expression of IL-10, and therefore have the potential to be used as a prophylactic and therapeutic agent in hypersensitivity and atopic disease [42, 43]. Recent studies have indicated that re-colonization of GI with appropriate strains of bacteria could localize lymphoid cell foci [43].

Lactic acid production and release of anti-pathogen substances by probiotics can help with immunity against infectious disease [43]. The heat-killed *Bifidobacterium breve* M-16 V could affect the expression of pro-inflammatory cytokines in the spleen and intestine [44].

Active Probiotics

Active probiotics have a crucial role in both innate and adaptive immunity because of their effects on dendritic cells [7, 45]. Dendritic cells act as a regulator of T-cell function by releasing

IL-10 and TGF-β [45]. A growing body of evidence has shown different immunomodulatory effects of probiotics on T cells [37].

Based on recent research, probiotic effects are as follows:

1. Modulation of the activity and composition of gut microbiome

   Most probiotics have nutrients competition with pathogenic bacteria. Moreover, some lactic acid bacteria are considered as probiotics [46].
2. Previous studies have shown that lactic acid bacteria could bind heavy metals such as cadmium [47].
3. The physical binding capacity of particular strains of probiotics can aid toxin removal, while acid and heat treatment could affect probiotics binding to toxic reagents [47, 48].
4. Probiotics can target signaling cascades occurring at the GI tract epithelium and thereby affecting immune responses through releasing soluble-factors [49, 50].

Immunomodulatory Effects of Probiotic Bacteria

Investigations on GF animals demonstrate that expansion of some mucosa-associated immune system is dependent on the bacterial colonization [50, 51]. However, it is not clear yet which components of bacterial cell wall are involved in maintaining the balance between the host and intestinal microbiota [50].

Cross talk between non-pathogenic bacteria and the host cells is mediated by signaling pattern recognition receptors, importantly TLRs. More precisely, lipoteichoic acid of gram-positive bacteria can activate immune responses through TLR2 [31], and peptidoglycan from both gram-positive and gram-negative bacteria can activate intracellular receptor (nucleotide-binding oligomerization domain-containing protein (Nod) 1/Nod2) [32]. Moreover, *B. fragilis* could activate the maturation of zwitterionic surface polysaccharide PSA [32]. In addition to the effects of soluble factors induced by probiotics, bacteria-specific unmethylated CpG motifs of the microbiome DNA is related to the fundamental differences between them [52]. For example, *B. vulgatus* inhibits IL-8 secretion from epithelial cells and systemic TNF-α production, thereby ameliorating inflammation in IL-10 knockout mice [52]. In addition, in mouse dextran sodium sulfate-induced colitis model chromosomal DNA of *B. vulgatus* was responsible for TLR9 signaling and its anti-inflammatory effects [33].

As mentioned above, probiotic bacteria components (cell wall, chromosomal DNA, and soluble metabolites) can regulate immune responses [50].

Probiotics and Autoimmune Diseases

In general, in many humans and animal models, probiotic administration is followed by the modulation of regional and systemic immune responses [53, 54]. Precisely, orally administered probiotic compounds prevent autoimmune diabetes by increasing pancreatic expression of IL-10 in non-obese diabetic mice [54]. Probiotic-induced soluble products could inhibit the production of inflammatory cytokines and affect epithelial ubiquitination and dendritic cells [55, 56]. Interestingly, different species of *lactobacilli* differentially influence dendritic cells; all species are able to induce IL-10 production at high concentrations but the ability to provoke IL-12 production is associated with a number of species [57].

In mice model, *L. acidophilus* CMUL067 could restore barrier integrity of epithelial cells and alleviate inflammation and colitis [58]. In addition, an association has been confirmed between IBD and leaky gut syndrome. Both correlate with an aberrant T_H1/T_H17 ratio [59]. However, probiotics can be helpful against IBD progression by increasing epithelial integrity, reducing TLR4 signaling, and inhibiting NF-κB expression [58].

Accumulating evidence suggests that the interaction between the host immune system and commensal microbiota leads to the inhibition of IL-17 and IL-22 production and therefore autoimmune disease modulation [60]. In an animal model of antiphospholipid syndrome (APS), probiotic fermented milk products have the capacity to modulate immunity by suppressing the level of autoantibodies [61]. Another study, performed at

Harvard Medical School University, by Tankou, SK, and colleagues, in 2018, demonstrated that administration of probiotics could alleviate allele HLA-DQA1 expression in MS and had a synergistic impression with contemporary MS therapies [62]. Moreover, in a pilot study, it is demonstrated that probiotic supplementation with *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species reduced mean fluorescence intensity (MFI) of CD80 on classical monocytes and diminished dendritic cell leukocyte antigen–antigen D related (HLA-DR) MFI [63]. In addition, research in EAE has revealed that commensal bacteria and their metabolites through interaction with G-protein-coupled receptors or inhibition of histone deacetylases affect T-cell-mediated autoimmunity [64].

Dietary Factors, Gut Microbiota, and Immunity

Dietary factors can influence gut microbiota composition at early and late stages of aging thus serving as a promising target for manipulating the gut microbiota to boost the immune system at every stage of life.

Fiber

As mentioned in the Introduction, anaerobic bacteria account for a major share of the total gut microbiota. These bacteria including *Bifidobacterium* and *Bacteroides* play an important role in dietary fiber fermentation with the final product of short-chain fatty acids (SCFAs). SCFAs serve as a source of energy for colonic and other peripheral tissues [65] and thereby play a role in shaping the interplay between the gut microbiota and the host [66]. Also, SCFAs have the ability to affect the colonic expression of genes involved in host metabolism [67] as well as immune responses [26]. For example, butyrate is a SCFA that has been shown to induce colonic T_{regs} and thereby may prevent colitis [68]. Along with the composition of gut microbiota, the amount of carbohydrates we consume will determine the quality and quantity of intestinal fermentation and associated byproducts, SCFAs [65].

Feeding GF mice a diet containing high lipopolysaccharide (LPS), a principal component of gram-negative bacteria, elevated the numbers of Mesenteric lymph node T cells (MLNs) and cellularity of Peyer's patches (PPs). The effect became more pronounced in conventionally colonized mice [69]. Overall, both LPS-rich diet and gut microbiota appeared to enhance the development of GALT), expansion of $CD4^+$ T cells expressing Foxp3 in MLNs and spleen, and production of T_H1 promoting cytokine IL-12, while decreasing the expression of T_H2-promoting cytokine IL-4. Further, conventional colonization, but not LPS-rich diet, was correlated with an increased expansion of $Foxp3^+$ $CD8^+$ T cells in PPs and MLNs and expression of anti-inflammatory cytokines IFN-γ and IL-10 in the spleen. In contrast, both LPS-rich diet and gut microbiota were shown to decrease the numbers of $CD19^+$ B cells in MLNs.

When compared to mice on a low-fiber diet, mice on a standard diet were resistant to allergic airway inflammation, as characterized by decreased infiltration of inflammatory cells (eosinophils and lymphocytes) in the airways, lower measures of inflammatory cytokines (IL-4, IL-5, IL-13, and IL-17A) in the lungs, and reduced secretion of antibodies (IgE and house dust mite extract (HDM)-specific IgG1) [70].

Fat

Each of high-fat diet and antibiotic therapy is sufficient to profoundly affect the gut microbiota composition [71]. The effect is further pronounced when both co-exist. More clearly, the percentage of gut microbiota similarity between chow-fed animals and chow-fed animals treated with antibiotics for 4 weeks was estimated to be 44%, while it was reduced to 22% between high-fat-fed animals and high-fat-fed animals treated with antibiotics for 4 weeks. In particular, high-fat diet reduced the content of bacteria belonging to the *Lactobacillus*, *Bifidobacterium*, and

Bacteroides-Prevotella genera. Moreover, compared to the WT mice, Leptin-deficient (*ob/ob)* mice, a genetic model of obesity and diabetes, reveal relative higher content of *Firmicutes* and lower content of *Bacteroides* in the caecal microbiota. Overall, adiposity would dynamically change contents of *Bacteroides* and *Prevotella*; the higher the calorie intake, the lower the contents of *Bacteroides* and *Prevotella* (for review see [65]).

High-fat feeding through increasing gut permeability causes metabolic endotoxemia, which is defined as an increase in plasma LPS concentrations by high-fat feeding. Metabolic endotoxemia, in turn, would mediate adipocyte hypertrophy associated with high-fat diet. Also, high-fat diet increased the expression of pro-inflammatory cytokines IL-1 and TNF-α in visceral adipose tissue. Thereby, feeding a high-fat diet led to lower glucose tolerance and higher glucose-induced insulin secretion, insulin resistance index, body weight gain, total energy intake, and visceral and subcutaneous adipose weight in mice [65].

Emulsifiers

Mice treated with dietary emulsifiers, carboxymethylcellulose (CMC) and polysorbate-80 (P80), revealed altered composition of microbiota in favor of increased content of *Bacteroidales* and decreased content of mucolytic bacteria such as *Ruminococcus gnavus* [72]. Studies of wild type (WT) and mutant mice showed that the dietary emulsifiers can contribute to the different degrees of colitis according to the genetic background: low-grade colitis in WT mice and high-grade colitis in IL-10$^{-/-}$ and TLR5$^{-/-}$ mice. Particularly, IL-10$^{-/-}$ mice which developed severe colitis had high relative contents of *Bilophila* and *Helicobacter*. Thereby, both emulsifiers led to development of metabolic syndrome as characterized by worsening glycemic control and increasing food consumption, overall weight, and adiposity. This might be, at least in part, due to altered gut microbiota composition as reflected in changes in SCFAs, which their anti-inflammatory actions are discussed above [72].

Iron Sulfate

Feeding the genetically susceptible (TNF$^{\Delta ARE/WT}$) mice with an iron sulfate containing diet was sufficient to cause severe inflammation in the distal ileum [73]. Also, no inflammation was evident in ileal tissue of both WT and TNF$^{\Delta ARE/WT}$ mice treated with intraperitoneal injections of ferric nitrilotriacetate (FeNTA). A proposed mechanism for the action of iron sulfate is via induction of endoplasmic reticulum (ER) stress leaving IECs more susceptible to CD8$^+$ T-cell (T_C)-induced apoptosis. Conversely, when iron was not included in the diet, TNF$^{\Delta ARE/WT}$ mice displayed no evidence of ileal inflammation. This might be mediated by altered gut microbial composition [73]. Luminal iron sulfate deprivation led to an increase in the relative content of bacteria belonging to the genera *Bifidobacterium*, *Succinivibrio*, *Turicibacter*, and *Clostridium*, while decreasing the content of bacteria belonging to the genera *Desulfovibrio* and *Bacteroides* [73].

Polyphenols

Polyphenol-rich extracts from cranberry are able to exert protective effects against weight gain in mice fed with high-fat high-sucrose diet (HFHSD), as reflected in increased energy efficiency and insulin sensitivity and decreased triglycerides (TG) and cholesterol content [74]. Cranberry extracts ameliorated inflammation and metabolic endotoxemia and improved antioxidative responses in the liver. More interestingly, these effects were accompanied with an action of cranberry extracts to alter the composition of the gut microbiota in terms of increased content of bacteria belonging to the phyla *Firmicutes* and *Verrucomicrobia* (especially the genus *Akkermansia*) and decreased content of bacteria related to the *Bacteroidetes* phylum [74].

Gut Microbiota Composition Is Altered in Immune-Mediated Disorders

Altered composition of gut microbiota, also known as dysbiosis, accompanies or even precedes the development of allergy [75], asthma [76], inflammatory diseases [77, 78], and infections [79, 80]. Precisely, low gut microbiota diversity in infancy would predict high risk of asthma at school age [76], and patients with psoriatic arthritis or skin psoriasis harbor a rather low diverse microbiota like patients with inflammatory bowel disease [78]. It is therefore possible with these data to propose that cleanliness of the intestinal environment can interfere with proper functioning of the immune system, providing the way for development and progression of immune-mediated disorders, known as the hygiene hypothesis (for review see [81, 82]). Supporting this is the improvement of allergy and inflammation with gut microbiota supplements [75].

In contrast, patients with chronic untreated human immunodeficiency virus (HIV) [79] and people infected with helminthes [80] sustain an overall high diverse microbiota.

Overall, an altered composition of gut microbiota can reflect health problems. Moreover, evidence of its discriminatory value comes from reduced diversity of gut microbiota in patients recently infected with HIV compared to patients with chronic untreated HIV [79].

Gut Microbiota Is Capable of Being Educated

Colitis Is Transmitted on the Background of an Endogenous Gut Microbiota

Mice lacking the transcription factor T-bet on a Rag-deficient background (*T-bet*$^{-/-}$ × *Rag2*$^{-/-}$) develop colitis resembling ulcerative colitis (the so-called TRUC model) [83]. Compared to that in *Rag2*$^{-/-}$ mothers, the gut microbiota in the offspring of conventionally colonized TRUC mice included increased proportion of *Bacteroidetes* and decreased proportion of *Clostridiales* and *Proteobacteria*. Using a series of experiments, the authors concluded that an endogenous microbial community is crucial to the development of UC and identified two *Enterobacteriaceae*, *Klebsiella pneumoniae* and *Proteus mirabilis*, as the most likely contributing bacteria in this context [83].

When Gut Microbiota Gets Education, It Can Be for a Long Time

The role of invariant natural killer T (iNKT) cells has been implicated in the development of inflammatory diseases such as Inflammatory Bowel Disease (IBD) and asthma. iNKT cells are innate immune cells able to recognize glycolipid antigens presented by MHC-like molecule, called CD1D, and induce the production of inflammatory cytokines IL-4 and IL-13. Oxazolone is used as a model of colitis where IL-13 release from CD1D-restricted iNKT cells causes ulcerative colitis (UC)-like features. Compared with specific-pathogen-free (SPF) mice, germ-free (GF) mice show an enduring difference in terms of greater numbers of iNKT cells in the LP of the colon and therefore are more susceptible to oxazolone-induced colitis and related morbidity and mortality [23]. As expected, treatment of GF mice with anti-CD1D antibody could inhibit oxazolone activity in the gut. Interestingly, this effect was also observed and sustained for two months when SPF mice gut microbiota were transferred into GF mice during neonatal period, but not during adult life. These observations were confirmed in the lung tissue as well and suggest a critical period for normal acquisition of gut microbiota [23].

The Immune System Mediates the Effects of Intestinal Microbiota on Extra-Intestinal Organs: Focus on the Gut–Brain Axis

Emerging evidence on the effects of the gut microbiota on the brain development, function, and behavior indicates the importance of immune factors as a key mediator in this regards (for review see [84, 85]). The study of gut microbiota

in neurological and psychiatric disorders is of recent origin. However, it has been promising enough that researchers have opened discussion to consider gut microbiota as a target for research and treatment of neuropsychiatric disorders such as major depressive disorder [86] and schizophrenia [87].

Higher secretion of IgA suggests an enhanced response to the lipopolysaccharide (LPS) of commensal bacteria (*Enterobacteriaceae*) in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) [88]. More interesting was the association of IgA responses with the activation of cell-mediated immunity and disease severity [88].

Thy1-aSyn (also known as ASO, alpha-synuclein (aSyn)-overexpressing) mice can be used as a model to study Parkinson's disease (PD). The authors in the study [89] evaluated germ-free (GF)-ASO mice and specific pathogen-free (SPF)-ASO mice with a complex microbiota in the comparison with GF-ASO mice. There was a relatively early onset of motor decline and decrease fecal output (especially water content) in SPF-ASO mice. This is consistent with the observation that motor decline is accompanied by the functional decline of the gastrointestinal (GI) tract in patients with PD. More interestingly, both the overall theme of aSyn aggregation and neuroinflammatory responses (microglia activation and production of pro-inflammatory cytokines IL-6 and TNF-α) were noticeably exacerbated in SPF-ASO mice. Post-natal antibiotic therapy effectively provided beneficial effects on motor and GI function in SPF-ASO mice. SCFAs can cause neuroinflammation and motor deficits and thus might be at least in part responsible for the involvement of gut microbiota in PD. Supporting this, studies provide evidence of altered SCFA content in microbiota of patients with PD [89].

Maternal immune activation (MIA) model is a model of autism where maternal infection increases the risk of autism spectrum disorders (ASD) in offspring. The study showed that MIA causes an impairment of intestinal integrity, so-called leaky gut [90]. It appears as early as from the third week of age and alters the expression of genes of which some are involved in immune responses. High levels of IL-6 as well as low levels of IL-12p40/p70 are present in MIA offspring. In addition, MIA offspring reveal altered gut microbiota composition mainly involving bacteria related to *Clostridia* and *Bacteroidia*. *B. fragilis* transfer at weaning has the ability to restore intestinal integrity, normalize IL-6 levels, resolve changes in gut microbiota, and attenuate behavioral abnormalities. Interestingly, in addition to the influence on the intestinal integrity, the maternal gut microbiota can affect the expression of tight junction proteins and thereby altering blood–brain barrier permeability in offspring [91].

The Gut Microbiota Mediates the Role of Immune System in Developing Diseases: Focus on Colorectal Cancer

As the name implies, colitis-associated colorectal cancer refers to the development of colorectal cancer on the background of intestinal inflammation. Recent evidence has suggested that the role of inflammation in cancer crucially involves alteration of composition of gut microbiota. In the study [92], IL-10-deficient mice were used as model for experimental colitis. When GF mutant mice were introduced to a SFP environment, all developed colitis, and that most of SFP mutant mice developed colon cancer after AOM administration. Analysis of microbiota composition revealed an overall reduced abundance of microbes in mutant than WT mice. Absence of such a difference between mice treated and not treated with Carcinogenic Agent Azoxymethane (AOM) would highlight the role of inflammation rather than cancer as a cause of altered composition of microbiota in the context of colitis-associated colorectal cancer. However; in mutant mice, inflammation increased relative content of bacteria belonging to the phyla *Verrucomicrobia*, *Bacteroidetes*, and *Proteobacteria*. In particular, the luminal abundance of *E. coli* was approximately 100-fold higher in mutant mice than WT mice, while, again, mice treated and not treated with AOM did not differ from each other in this respect.

Studies have confirmed the presence of altered microbiota in both patients with colorectal cancer and colitis [93].

Gut Microbiota: Role in Cancer Immunotherapy

Gut microbes can cooperate with chemotherapy and immunotherapy drugs in order to trigger stronger anti-cancer immune responses.

Germ-free and ACS-treated mice display reduced effectiveness of CTLA-4-specific antibody as reflected in decreased activation of effector $CD4^+$ T cells and tumor-infiltrating lymphocytes (TILs). To clarify the role of gut microbiota in effective cancer immunotherapy, Vétizou and colleagues (2015) [19] used a subclinical colitis model, where CTLA-4-specific antibody through a T-cell dependent manner induced cell death and IEC proliferation in the ileum and colon. When a combination of *Bacteroides* administered to germ-free or ACS-treated mice, the efficacy of CTLA-4-specific antibody was increased in terms of intratumoral DC maturation and IL-12 signaling-dependent T_H1 responses in tumor-draining lymph nodes (TDLN) [19].

Administration of non-myeloablative doses of cyclophosphamide (CTX) to naïve mice resulted in villus atrophy in small intestine, interstitial edema, mononuclear cell infiltration in LP, and an increase in permeability of the intestinal mucosa [94]. CTX is able to direct translocation of gram-positive commensal bacteria (*Lactobacillus johnsonii*, *Lactobacillus murinus*, and *Enterococcus hirae*) into the MLN and spleen (by more than 50%), and it causes dysbiosis of microbial composition of the small intestine (decreased content of bacteria belonging to the genera *Clostridium cluster XIVa*, *Roseburia*, *unclassified Lachnospiraceae*, and *Coprococcus)*. Dysbiosis accompanied with decreased frequency of $CD103^+$ $CD11b^+$ dendritic cells and $TCR\alpha\beta^+$ $CD3^+$ $ROR\gamma t^+$ T cells, which are involved in inducing T_H17 cells, in LP of the small intestine as well as with increased polarization toward T_H1 and T_H17 cells. Interestingly, treatment with antibiotics for gram-positive organisms hindered the anti-cancer actions of CTX, reducing polarization toward Th17 cells, infiltration of pathogenic Th17 cells in the spleen, and intratumoral infiltration of $CD3^+$ T and Th1 cells. Finally, the authors showed that the anti-cancer effects of CTX in GF mice by colonization with SFB [94].

Conclusions

The current decade has witnessed an increasing interest in studying the convergence between dietary factors, gut microbiota composition, and immune responses. The present chapter provides evidence that not only intestinal immunity but the total body's immune system would be influenced by the composition of the gut microbiota. Supporting data come from animal studies where specific pathogen-free (SPF) microbiota could contribute to progression or healing of the intestinal and extraintestinal health problems including allergy, asthma, inflammatory disorders, and cancer. On the other hand, Certain immune cells deficient mice which are genetically susceptible to spontaneous development of diseases display dysbiosis of the gut microbiota. In this manner, there is a mutual relationship between the gut microbiota and the immune system. Environmental factors in association with genetic predisposition play a role in shaping this relationship. Dietary factors are environmental factors most amenable and, of course, appropriate to optimization that can be used throughout life to concurrently change the composition of the gut microbiota and immune responses.

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Maternal Nutrition, Child Development, and Immunity

11

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Key Points

- The mother provides passive immunity through the transplacental and lactation routes.
- Maternal nutritional status plays a role in determining the ontogeny of the fetal immune system.
- Maternal stress can adversely affect the developing immune system of the offspring.
- Maternal nutritional status, maternal stress, and microbiota can influence the risk of developing diseases, such as inflammatory and autoimmune diseases, allergy and atopic diseases, and cardiometabolic diseases in the offspring.
- By manipulating nutritional status and maternal stress, it might be possible to hinder the progression of diseases in the offspring.

Introduction

About seven million children under the age of 5 years died in 2011 [1]. Infant mortality has grown into a serious global health issue. This is majorly due to the suboptimal response of infants to infections and vaccinations. The fetal and neonatal period is characterized by immunological immaturity, while epigenetic and environmental factors such as maternal nutrition intervene to define its course. Moreover, a recent hypothesis of immune suppression during the fetal and neonatal period has been suggested [2]. Neonatal immunodeficiency is a consequence of an essential adaptation during the progression from fetal to neonatal stage which might take several years after birth to overcome [3]. It is characterized by suppressed T cell-mediated responses, immature B and T lymphocytes, poor cytokine and antibody production, and repressed natural killer (NK) cell activity. Consequently, the infant is susceptible to infections. Furthermore, this is more severe and chronic in preterm neonates, which explains the high risk of morbidity, sudden death, and neurologic sequelae [4]. However, passive immunity provided by the mother during pregnancy and lactation compensates and concerts with the developing immune system of the infant to fight against potential pathogens. Therefore, maternal nutritional and psychosocial statuses play a role in defining the strength and development of the infant immune response.

Fetal and Neonatal Immature Immune System

The immune system of the neonate is characterized by limited number and specificity of lymphocytes, incomplete oral tolerance, and scarcity in T regulatory lymphocytes [5]. Cell-mediated immunity is regarded as immature during the fetal and neonatal period with a shift of T helper (T_H)-1 cell/T helper (T_H)-2 cell balance toward T_H2 cell response. This is due to delayed maturation of interleukin (IL)-12- and type I interferon (IFN)-producing dendritic cells (DC) [6]. These have the capacity to produce cytokines, such as IL-18, but at levels impotent of activating NK cells [7]. The latter are the lymphocytes that are capable of killing infected cells in a major histocompatibility complex (MHC)-independent pathway in the absence of previous sensitization [8]. In the fetal period, NK cells are immature $CD56^+$ and $CD56^-$ cells with impaired viral suppressive activity, characterized with weak cytotoxic function and reduced expression of IFN-γ and other cytokines [2]. Moreover, neonatal $CD4^+$ T cells show downregulation in the expression of a number of cytokines, including IL-4, IL-5, and IFN-γ, rendering these cells poorly responsive to physiological stimuli [9]. Several mechanisms have been suggested, including poor signal transduction pathways, low volume of effector/memory cells (low levels of IL-4 and IFN-γ), and production of immature DCs with reduced expression of IL-12 [2]. Neonatal T lymphocytes also exhibit reduced expression of perforin, weakening their cytolytic ability [2]. With respect to the humoral immune response, T cell-independent response, directed against polysaccharide antigens, is absent until the third month of age, reaching maturity at the age of 4 to 5 years. This is mainly due to the immature splenic marginal zone architecture and low expression of CD21. However, T cell-dependent response, directed against protein antigens, partially overcomes the situation through toll-like receptor (TLR)-9 signaling, whereby B

cells acquire $CD27^+$ IgM^+ phenotype and differentiate into plasma cells capable of producing Immunoglobulin (Ig)-M. Nevertheless, T cell-dependent pathway is not that mature too, due to the downregulation of the CD40 ligand on $CD40^+$ T cells, delayed maturity of follicular DC, restrained antibody affinity maturation as a result of reduced somatic hypermutation, the absence of stromal cell support, and impaired priming of effective T_H responses [2]. All these contribute to a weak immune system incapable of fighting potential pathogens, putting the fetus or the neonate at high risk of morbidity and mortality.

Fetal and Neonatal Suppressed Immune System

Several regulatory mechanisms govern the immune system of the fetus to ensure tolerance by preventing inflammatory responses with the mother; otherwise, miscarriage or fetal resorption might occur. The first mechanism is through regulatory T (T_{REG}) cells that respond to specific antigens and strongly suppress T cell proliferation [2]. Levels of T_{REG} cells begin to decrease near delivery, reaching adult levels at birth [10]. Second, upon TLR stimulation and suppression of IL-12 production by DCs, $CD5^+$ B cells or B-1 cells release IL-10 favoring T_H2 cell response. B-1 cells are important as the first line of defense against pathogens due to its low-affinity IgM antibodies. They comprise approximately 40% of B cells at birth and continue to increase until 4 months of age where beyond this point they gradually decrease [2]. Myeloid-derived suppressor cells (MDSC) also play a role in regulating the fetal and neonatal immune system. These cells are usually low in number but increase whenever chronic inflammation exists. MDSCs have been shown to suppress T cell activation and proliferation, decrease the release of IL-5, IL-17, and IFN-γ [11], push toward T_H2 cells response [12], and inhibit NK cell cytotoxicity [13]. The key player of the fourth mechanism is the mesenchymal stromal cells (MSC), which are multipotent cells, capable of regulating the immune system. They can suppress monocyte differentiation into immature DC [14], $CD8^+$ T cells [15], naïve and memory T cells [16], and NK cells [17], indirectly promote functional $Foxp3^+$ T_{REG} cells, and favor T_H2 cell response [2]. The aforementioned mechanisms are hypothesized to collectively suppress the immune system of the fetus and their effect continues for a while after delivery.

Epigenetic and Environmental Factors Affecting Fetal and Neonatal Immune System

Prenatal and early postnatal growth rate influence a number of diseases in adulthood, including stroke, coronary heart disease, chronic obstructive pulmonary diseases, hypertension, type II diabetes mellitus, dyslipidemia, and blood coagulation disorders. This is "fetal and neonatal origins of adult disease hypothesis," which was first proposed by Barker in 1994 [18]. Scientists agree that nutritional programming during gestation might permanently affect the immune system [19]. One example is the "thrifty phenotype," where the metabolic response to undernutrition during the fetal period would be "inappropriate" during overnutrition, later in life, leading to disease manifestation [20]. This was observed in studies done in rural areas where seasonality (hunger season vs. harvest season) was prevalent and mortality rates reached significantly high levels among infants during hunger season [19].

Lymphoid tissues are highly demandable, rendering them extremely vulnerable in case of nutritional deficiencies. Therefore, both immunological competency and nutritional status are major determinants of morbidity and mortality [21]. On the short term, protein-energy malnutrition (PEM) impairs host defenses resulting in reduced cell-mediated immunity and NK cell activity [22–24]. On the long term, permanent thymus involution, known as "nutritional thymectomy," has long been observed [25]. The ontogenetic development of the immune system would be influenced by environmental factors, such as nutrition and infection, particularly during the fetal stage. Therefore, intrauterine malnutrition would lead to more severe and chronic abnormalities than those occurring later in life [26]. Infants are already born with suboptimal immune functioning, which would be exacerbated in *small for gestational age* infants, with thymic atrophy, impaired cell-mediated immunity, and hypoimmunoglobulinemia (particularly, IgG) [24]. However, premature

infants show worse immunocompetence which persists up to 9 months of age [27].

Lactation, Milk Composition, and Immunity

In the 1950s, improving infant formula composition was on the run and many turned to artificial feeding as a 'better substitute' of human milk, particularly with the advantage of better weight gain observed in formula-fed infants [28]. However, studies have shown that exclusive breastfeeding for at least the first 6 months of life has various beneficial outcomes on the short and the long term, such as reducing the risk of gastrointestinal diseases, allergies, otitis media, and respiratory infections [29–31]; reducing the risk of sepsis, necrotizing enterocolitis, and mortality [32]; and better IQ scores [33]. Breastfeeding reduces the risk of atopic diseases by limiting exposure to food antigens, aiding in the maturation of the neonatal intestinal mucosa by providing easily absorbed nutrients, protecting against infections, and providing anti-inflammatory components and antibodies [34].

"Breast milk is the best nutrition for infants" [35]. It ensures the optimal development and growth for the infant by providing balanced nutrients adjusted to its growing needs and protection against the vast array of pathogens through redundant and synergistic components of both adaptive and immune response. The protective components of milk include prebiotics that can promote the growth of commensal bacteria, multifunctional components with anti-inflammatory and immunomodulatory activities, glycans, and pathogen-specific secretory immunoglobulins. The collective action of these components, along with the developing immune system of the infant, provides the essential protection against any potential pathogen [28].

Milk Oligosaccharides and Other Glycans

Human milk contains oligosaccharides (complex carbohydrates with lactose moiety), glycolipids (complex carbohydrates attached to lipid molecule), glycopeptides, glycoproteins, glycosaminoglycans, and mucins. These glycans afford the infant robust protection by competitively inhibiting the pathogens from docking to host cell receptors [28], such as labile toxins of *Escherichia coli* and *Vibrio cholerae* [36], *Streptococcus pneumonia* [37], enteropathogenic *Escherichia coli* [38]. The oligosaccharides influence the composition of the microbiota of the infant gut, which might be the reason behind the fact that breastfed children have less potentially pathogenic *Escherichia coli*, *Klebsiella*, and other *Enterobacteriaceae* strains than formula-fed children [39]. Group B *Streptococcus* or *Streptococcus agalactiae* is the leading cause of neonatal infection, vertically transmitted from the mother to the newborn because of the maternal vaginal colonization. A recent paper unveiled a role for human milk oligosaccharides in protecting against this infection by impairing growth kinetics of Group B Streptococcus [40].

Glycoconjugates also have anti-adhesion effects. For instance, milk glycolipid Gb3 inhibits the adhesion of *Shigella dysenteriae* and Shiga-like toxin of enterohemorrhagic *Escherichia coli* [41]. Glycoprotein Mac-2 aids the macrophages in binding to the pathogens as the step preceding phagocytosis. Mucin intercepts to inhibit *Escherichia coli* from binding to epithelial cells and to prevent rotavirus from replicating. Casein components also prevent mucosal attachment of *Actinomyces* and *Streptococcus* species [42].

Milk Serum Proteins and Immune Cells

Milk serum proteins are the most abundant milk proteins (60% of total proteins), comprising essential amino acids, coenzymes, and bioactive proteins, which play a key role in the maturation of the immune and digestive system in early life. These types of proteins are qualitatively the same among mothers but change quantitatively over the course of lactation, depending on the needs of the growing neonate [35]. In the first class of milk serum proteins, enzymes, there are two highly abundant proteins, α-lactalbumin (LALBA) and bile acid-activated lipase (CEL). LALBA is a lactose synthase subunit and a potent

apoptotic agent with a key role in the immune system [43, 44]. CEL is necessary for fat digestion and absorption when incorporated with gastric lipase and is able to withstand the acidic medium of the stomach until it is activated in the small intestine [45]. CEL is relatively abundant during the first 2 weeks of life and then decreases at the 24th week, when the digestive system has reached its maturity [35]. The second class of milk serum proteins is nutrient transport proteins, which vary considerably over lactation. Serum albumin (ALB) is a dominant transport protein, which provides amino acids and binds to zinc, copper, and thyroxine. Fatty acid-binding protein 3 (FABP-3) transports long-chain fatty acids, facilitating their esterification into triglycerides. ALB and FABP3 significantly increase over lactation, indicating their necessity for nutrient absorption [35]. On the other hand, there are other nutrient transport proteins that are decreased significantly over lactation, indicating their importance during early stage [35]. These include serotransferrin (TF), which has an antibacterial effect by shuffling iron into the cell [46], and Selenium-binding protein 1 (SELENBP-1), which conveys selenium into the cell as a cofactor for antioxidant enzymes [47]. The third class comprises the immunity proteins, which include the immunoglobulins and lactotransferrin (LTF). These ensure immune protection for the infant on both the innate and adaptive levels of the immune response [35]. IgA is the most abundant antibody in human milk, providing antigen-specific protection for the gastrointestinal system against pathogens [48]. On the other hand, LTF, CD14, complement proteins, and protease inhibitors, among many others, provide a rapid and ongoing protection for the infant [35]. LTF has bactericidal effect by differential glycosylation and chelating free iron [49, 47] and reduces pro-inflammatory cytokines by the monocytes [50]. CD14 helps the innate mucosal immune system to recognize gram-negative bacteria [51]. Complement components are necessary for the destruction of bacteria and the neutralization of viruses. Protease inhibitors, such as inter-α-trypsin inhibitor heavy chain H2 (ITIH2) and α-1 antitrypsin (SERPINA1), regulate the complement system and protect the immunoglobulins against degradation [52, 53]. Breast milk is an important source of Pentraxin-3 (PTX3), protecting neonatal mice against *Pseudomonas aeruginosa* pulmonary infection [54]. Prototypic long PTX3 is a soluble protein receptor with pleiotropic functions in the innate immunity and inflammation. PTX3 is produced by myeloid cells, endothelial cells, and epithelial cells, among others, and is released by neutrophils in response to pro-inflammatory cytokines and microbial antigens [55]. It would promote phagocytosis and clearance by binding to specific pathogens [56].

Colostrum and mature milk are better defined as "suspensions of viable cells in highly nutritive media" [57]. They significantly contain cells of hematogenous origin that have infiltrated the interstitial connective tissue, ductal, and alveolar epithelium of the breast right after parturition. The cellular infiltration decreases as the milk approach maturity. Milk cell count varies intra- and inter-individually, but is comprised mainly of epithelial cells, lipid droplet-laden macrophages known as colostrum corpuscles, polymorphonuclear neutrophils, B and T lymphocytes, and sometimes eosinophils [57]. Lymphocytes are naturally transferred to the neonate during lactation and are able to withstand the pH and the enzyme activity in the stomach. These would transverse the gastrointestinal lining and sensitize the neonate immune system [58] and provide immunological protection by transferring delayed hypersensitivity from the mother [59]. Macrophages viable in the breast milk offer protection against necrotizing enterocolitis, a disease affecting premature, low-birth-weight infants [60].

IgG is the only antibody that can cross the placental border. IgG secreted in milk enters the systemic circulation of the neonate via the neonatal Fc receptor (FcRn) found on the surface of the enterocytes [61]. Moreover, despite the fact that colostrum and milk do contain antibodies, none are absorbed by the infant gastrointestinal tract, such as Rhesus antibody and ABO isoagglutinins. Secretory IgA is the predominant immunoglobulin in milk and plays a vital role in protecting the mucosa against enteric microorganism such as *Escherichia coli*, the most common cause of neonatal meningitis [57]. The neonate is born deficient in IgA, the main trouper in defending the mucosal

membranes in the gastrointestinal, respiratory, and genitourinary tracts. IgA is transferred from the gastrointestinal mucosa of the mother to the mammary gland to be secreted in the milk along with other beneficial factors, such as lactoferrin, lysozymes, macrophages, and granulocytes. These would be effective in protecting the neonate against a number of pathogens, while, still, his own antibodies are scarce [62]. Milk provides defense for the infant without inducing inflammation that could be costly and cause impaired growth [5].

Infectious Agents in Milk

Breastfed infants are at increased risk of vertical transmission of certain viral infections from the mother, such as Human Immunodeficiency Virus (HIV) and Cytomegalovirus (CMV) [63]. Lactation is contraindicated in maternal HIV infection, unless there are no milk substitutes, as in developing countries [64, 65]. Despite this, most infants born to HIV-1-infected mothers do not contract the disease after birth, indicating that there are milk components that limit the vertical transmission of HIV-1. Pollara et al. suggested that mucosal HIV-1 envelope-specific IgA responses reduce the risk of transmission via breast milk [66]. Regarding CMV infection, risk must be "counterbalanced" against the vast benefits of breastfeeding [67]. However, other infections cannot be vertically transmitted and lactation is encouraged. Nevertheless, appropriate measures should be taken in case of infection, such as immune prophylaxis in case of hepatitis, antimicrobial therapy in case of *Mycobacterium tuberculosis* and *Haemophilus influenzae* infections, and temporary cessation of lactation for a limited time in case of *Neisseria gonorrhea* (for 24 hours) [63].

Maternal Malnutrition

Nutrients are essential in modulating the developing immune system of the neonate [68], thereby dictating the future risk of developing a number of diseases such as cardiovascular diseases (CVD), type II diabetes mellitus, and some cancer types. Prenatal and postnatal nutritional status of the mother is a determinant factor in the organ development of the offspring and has a huge impact on the pathogenesis of metabolic and mental diseases [69]. Maternal nutritional status before and during pregnancy dictates prenatal nutrition and is affected by maternal body index, stress, smoking, etc. Placental function and gestation duration are environmental factors that affect fetal development. Moreover, the postnatal nutritional status would depend on the maternal nutrition status in case of lactation, or type of formula, duration of lactation, and microbiota status [70]. Maternal malnutrition impedes placentation, thereby decreasing the transfer of nutrients, hormones, and immune factors to the growing fetus [71]. Studies have shown that perinatal nutrient deficiency results in thymic atrophy and reduced function in adulthood [72, 73], due to an interplay between genetic factors, neuroendocrine system, maternal factors, neonatal microflora, and antigen exposure [74–77]. Nutrients influence the innate immune signal transduction pathways and the maturity of immune cells, thereby imprinting initial sensitivity to antigens, oral tolerance, and host defense against pathogens [78]. Perinatal malnutrition results in lower thymopoietin levels (poor growth rate) [79] and poor humoral response [72]. Prenatal stress affects the hypothalamic-pituitary-adrenocortical (HPA) axis leading to reduced thymic function [80, 81], shorter length of gestation, lower birth weight [82], and impaired cytokine secretion [83]. The resulting hypercortisolemia is common in immunodeficiency [84]. Moreover, malnutrition suppresses leptin production, which, synergistically with elevated levels of glucocorticoid hormones, in turn, causes a reduction in the production of thymic hormones [85]. Malnourished neonates exhibit increased pro-inflammatory cytokine production [86, 87] and suppressed "compensatory" anti-inflammatory immune response [88], rendering them susceptible to increased inflammatory damage. Intrauterine malnourishment has been associated with an increased risk of lung allergic inflammation, such as asthma [89].

Maternal Undernutrition: Energy and Nutrient Deficiencies

The human neonate is born with an immature immune system, increasing the risk of infection during this critical period. However, transplacen-

tal IgG and antimicrobial proteins in human milk, such as IgA, complement compounds, and lysozymes, provide specific and nonspecific immunity in the gastrointestinal and possibly in the respiratory tracts of the neonate. Many studies investigated the effect of maternal nutritional status on the composition and volume of milk, showing deficiencies in water-soluble vitamins, minerals, protein, and fat and a decrease in milk volume. Moreover, colostrum of malnourished lactating mothers has been significantly low in IgG, IgA, and complement C4. While IgG and lysozyme are actively transported across the placenta regardless of maternal nutritional status, there was a specific influence of malnourishment during lactation on certain host defenses proteins, such as IgA and complements C3 and C4 [90].

Undernutrition is known to suppress immunity and increase the risk of infection in children, particularly those younger than 5 years of age. T cell function, secretory IgA, and complement system are significantly repressed during nutritional stress. Watson and McMurray showed that malnutrition results in increased phagocytosis and T cell function against cancerous cells while decreasing the ability of the immune system to fight a number of pathogens [91].

Maternal Energy-Protein Deficiencies

There is an increased attention for the effect of macro- and micronutrients during pregnancy on the aptness of the immune system of the neonate against the potential pathogens. In one animal study, maternal protein-energy malnutrition had no effect on the bactericidal effect of neutrophils of neonates [92]. However, another animal study demonstrated that both low (6.5% protein) and high (30% protein) protein-carbohydrate ratios in the diet fed during gestation period had negative effects on the immune function of the neonates on the short and the long term [93]. Immunoglobulins in the low-protein group (LP) and high-protein group (HP) were significantly lower in the one-day-old neonates when compared to the adequate-protein group (AP) (12.1% protein) ($P < 0.05$). This was attributed to the fact that the elevation of maternal cortisol level because of nutritional stress accelerated the fetal gut maturation, thereby limiting the absorption of colostral immunoglobulins. Tuchscherer et al. concluded that the effect of the impaired maternal nutritional status on the immunity of the offspring is mediated by the maternal glucocorticoids, as demonstrated by the elevated maternal cortisol levels. The nutritional stress during gestation has repressed the humoral immunity, as evidenced by the lower immunoglobulin concentrations in both LP and HP groups ($P < 0.05$), and the cellular immunity, as evidenced by the higher $CD4^+$ T cells and $CD4^+/CD8^+$ T cell ratio in HP group ($P < 0.05$), and accentuated cytokine responses to inflammatory stimulus, as evidenced by the higher IL-6 levels in both LP ($P = 0.09$) and HP ($P < 0.01$) groups and higher IL-10 in LP ($P < 0.05$) group when stimulated with lipopolysaccharide (LPS) [93]. These events predispose the offspring to a great risk for postnatal morbidity as a result of impaired defenses against possible pathogens. These findings were confirmed by a later study, where the effect of maternal moderate energy (40% below the requirements) or protein (40% below the requirements) restriction during late gestation on the immune function of the neonates was investigated [94]. The results revealed that the offspring of both groups had lower ($P < 0.05$) plasma concentrations of complement components (C3 and C4) and immunoglobulins (IgG and IgM) and lower mRNA expression of IL-2 and IL-6 in the jejunum when compared to the control group. This study agrees with many others, which suggest that malnutrition causes an impairment in thymocyte proliferation at birth and thymic and spleen lymphocyte proliferation at weaning [95]. This, in turn, prompts the production of deciduous and dysfunctional T lymphocytes [96] accompanied with delayed activation of nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) [97]. Thereby, the expression of cytokines such as IL-2, IL-6, and IL-10 is impaired [94]. Moreover, malnourishment disturbed the mucosal integrity of the small intestine as evidenced by a decreased mucosal thickness and villus height [94], which might be a consequence of impairment in the proliferation-apoptosis balance [98]. The upregulation of tight junction proteins, such as Zonula occludens-1 (ZO-1), ensures mucosal integrity, thereby reducing the risk of intestinal inflammatory disease. Animal studies showed that the maternal low energy diet

(3.0 MCal digestible energy/kg diet in comparison to control diet with 3.4 MCal digestible energy/kg diet) significantly decreased ZO-1 gene expression ($P < 0.05$) [99]. Moreover, there was a significant elevation in the levels of pro-inflammatory cytokines, such as IL-1β, IL-10, and tumor necrosis factor (TNF-α) ($P < 0.05$), in the activity of apoptotic transduction cascades, thereby leading to increased intestinal permeability [99]. These events created additional risk for infections and mortality since mucosal integrity is considered as a physical barrier against the microbes existing in the intestinal lumen. He et al. also observed that, when the offspring reached 6 weeks of age, their basal immune system revived from maternal malnutrition as no significant difference in plasma or tissue immune parameters was detected among the three groups. However, its influence on tissue cytokines expression in response to LPS lingered, causing an uncontrolled immunological response with the overexpression of IL-6. He et al. suggested this as the mechanism behind fetal origins of adult disease.

Developmental programming is a term used for the influence of environmental factors, such as nutritional factors, during the fetal and early neonatal periods on the health of the organism for the long term. The immune system of mammals develops during these two critical growth periods, dictating its future vulnerability to microbial pathogens. Once the immune system reaches its maturity, its aging process or immunosenescence begins. Thymus involution with a decrease in T cell output and a shift in the balance between the memory T cells and naïve T cells in the periphery are among the events that increase the individual susceptibility to cancers and infections associated with aging. Heppolette et al. studied the effect of maternal protein restriction during lactation on the immunosenescence of adult mice. Postnatal low-protein (PLP) diet (8% protein) was fed to dams during lactation, while the control group received normal protein requirements (20%) [100]. The PLP offspring exhibited slower growth ($P < 0.001$), yet a slower rate of thymic involution ($P < 0.001$) and splenic aging, when compared to the offspring of the control group. Moreover, PLP offspring showed improved T cell receptor signaling and T cell responsiveness, higher proportion of naïve $CD4^+$ and $CD8^+$ T cells, higher ratio of naïve to memory $CD4^+$ and $CD8^+$ T cells ($P < 0.05$), increased staining ($P < 0.01$) and density ($P < 0.05$) of germinal center (GC) sites, and lower p16 (a senescence-promoting tumor suppressor protein) gene expression ($P < 0.05$) in spleen [100]. Heppolette and coworkers concluded that maternal protein restriction might delay the aging of the adaptive immune system, posing a great potential for the lactation period in preventing age-associated diseases.

The development of secretory IgA in the saliva was investigated in 263 children up to the age of 5 years, along with cross-sectional studies in Australia and Papua New Guinea highland. The pattern of this development seemed to be influenced by several factors among which was nutritional status and feeding. The malnourished children exhibited less total IgA and specific IgA antibodies to *Escherichia coli* and *Haemophilus influenzae*, when compared to well-nourished children [101].

Most experiments analyzing the effect of malnutrition on immunosuppression were done on cell-mediated immunity, where there is an elevation of T cell to B cell ratio. In PEM, serum immunoglobulin levels are either normal or much elevated; however, in both cases, the produced immunoglobulins are polyreactive [102]. It seems that the effect of malnutrition on humoral immunity varies depending on the type of malnutrition. Energy restriction alters humoral immunity, characterized by the suppression of T_H1 and T_H2 cytokine-induced immunoglobulin production. Neyestani et al. investigated the effects of acute PEM on immunoglobulin isotypes and subclasses and found out that ratio of the sum of T_H2 to T_H1-type immunoglobulin (determined as IgG1 + IgG2b/IgG2a + IgG3) was higher in both energy restriction and low-protein animal models, indicating a systemic shift to T_H2 arm activation. Neyestani and coworkers suggested that this might be impaired during parasitic infection. Therefore, protein undernutrition or overnutrition is an effector player in immune function either ways [102].

Maternal Overnutrition: Overweight and Obese

Obesity has grown into a global pandemic, with approximately 66.9% of US women of reproductive age being overweight (body mass index [BMI] 25–29.9 kg/m^2) [103]. It is considered an inflammatory condition. Maternal obesity is associated with adverse outcomes perinatally and an increased risk of developing obesity, insulin resistance, hypertension, and CVD in offspring during adulthood [104]. As a host defense mechanism, inflammation renders essential nutrients unavailable for the invading pathogen, thereby reduced levels of several vitamins and trace elements are observed [105], which may, in turn, decrease the level of nutrients transferred to the offspring. Folate, cobalamin, vitamin B6, vitamin D, zinc, and iron are deficient in obese pregnant women and obese adults [106–109].

In the mother, increased BMI is associated with elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, depressed levels of adiponectin [110], and increased oxidative stress and nitrative stress in the placenta [111]. Oxidative stress occurs because of an imbalance between the production of reactive oxygen species (ROS) and the ability to scavenge these by antioxidant enzyme systems. The oxidative stress in obesity is further exacerbated by pregnancy due to the high metabolic activity of the placental mitochondria, producing ROS [112]. Elevated levels of ROS are observed in preeclampsia, diabetes, and intrauterine growth restriction [113, 114]. Nitrative stress can occur in the placenta as a result of the generation of peroxynitrite ($ONOO^-$), a potent prooxidant that would cause protein nitration, thereby leading to preeclampsia [114] and pregestational diabetes [113]. Roberts et al. found that increasing BMI in pregnant women is associated with increased oxidative and nitrative stress [111].

Infants born to obese women are more susceptible to birth difficulties, macrosomia, perinatal death [115–118], and congenital anomalies, including neural tube defects (odds ratio [OR] = 1.87; 95% confidence interval [CI], 1.62–2.15), spina bifida (OR = 2.24; 95% CI, 1.86–2.69), cardiovascular anomalies (OR = 1.30; 95% CI, 1.12–1.51), septal anomalies (OR = 1.20; 95% CI, 1.09–1.31), cleft palate (OR = 1.23; 95% CI, 1.03–1.47), cleft lip and palate (OR = 1.20; 95% CI, 1.03–1.40), anorectal atresia (OR = 1.48; 95% CI, 1.12–1.97), hydrocephaly (OR = 1.68; 95% CI, 1.19–2.36), and limb reduction anomalies (OR, 1.34; 95% CI, 1.03–1.73) [119]. Evidence shows that in utero environment plays a critical role in determining adult diseases, particularly dysmetabolic disorders such as hypertension, CVD, and diabetes mellitus. Maternal hypercholesterolemia and maternal immune mechanisms are two key factors in affecting the developmental programming of immune-mediated disease such as atherosclerosis, rendering the infant susceptible to accelerated progression towards inflammatory diseases even under normocholesterolemic conditions [26].

Maternal Micronutrient and Macronutrient Status, and Immunity

Maternal nutritional status is an important indicator for determining the micronutrient status of the infant. Morbidity and mortality rates of children under 5 years of age increase considerably when micronutrient-deficiency exists, with approximately two-thirds of global mortality due to infectious diseases [1]. Therefore, maternal supplementation is much emphasized.

Vitamins

Vitamin A

Vitamin A, particularly its active metabolite, (all-trans and 9-cis) retinoic acid (atRA), is an important micronutrient in the context of a healthy immune system [120, 121]. atRA seems to be the most important form for normal fetal development [122]. It maintains mucosal integrity [123], regulates the proliferation, differentiation, and cytokine secretion of various immune cells [124], and is involved in the thymic development and thymocyte maturation. It also plays an important role in the innate immune system, intestinal toler-

ance, immune system homeostasis [125], and humoral immunity [126]. Hypovitaminosis A impairs the functioning of lymphocytes, NK cells, and neutrophils and suppresses cell proliferation and immunoglobulin production [121, 124]. Vitamin A supplementation induces T cell proliferation and activation, particularly $CD4^+$ T cells [127]. It suppresses T_H17 cell responses and induces T_{REG} cells [128]. While vitamin A deficiency leads to a skew toward T_H1 cell response, vitamin A supplementation favors T_H2 cell response [129].

During gestation, vitamin A, mostly in the form of retinol, is transferred from the mother to the fetus across the placenta under tight regulation by the mother's homeostasis [130]. RA synthesis begins during early fetal development [122]. β-Carotene, the precursor of vitamin A, follows the transfer of vitamin E and seems much less effective than retinol in the transfer across the placenta [131]. The infant is born with low vitamin A status due to the tight regulation of placental transfer. Retinol-binding protein (RBP) synthesis starts only in the third trimester [132]. Therefore, the infant relies on milk ingestion to obtain its vitamin A requirements. Up to the age of 6 months, breast milk is the sole source of vitamin A in exclusively breastfed infants [133]. Moreover, vitamin A content varies depending on the maturity of milk and maternal dietary supplementation [134]. Colostrum contains thrice as much vitamin A and ten times as much β-carotene (precursor of vitamin A) than mature milk [133], indicating the importance of colostrum ingestion immediately after parturition.

However, vitamin A deficiency is a great problem in developing countries, increasing morbidity and mortality rate among children. Timing seems to exert a greater effect on vitamin A metabolism, immunomodulatory effect, and transfer to mammary glands, when compared to dosage. A high dose of vitamin A supplementation did not result in any significant increase in vitamin A content of colostrum [135]. Despite the WHO/UNICEF/IVACG Task Force recommendation of postpartum vitamin A supplementation (400,000 IU divided into two doses, at least a day apart) for those with depleted stores, there was no effect on maternal and infant morbidity and mortality rates [136]. Consequently, 2011 WHO guidelines did not recommend postpartum vitamin A supplementation.

On the other hand, vitamin A supported the immune response to hepatitis B vaccine in neonates [137] and reduced risk of fever and malaria episodes in children when combined with zinc [138]. Moreover, plasma retinol concentrations of 2-month-old infants inversely correlated with a positive skin test at 5 and 20 years of age and with allergic diseases at 20 years [139]. An inverse correlation was also observed between mucosal integrity and serum retinol levels [140]. Vitamin A deficiency consociates with the burden of infections and it is a risk factor for vertical transmission [78].

Vitamin D

Vitamin D plays a vital role in the regulation of calcium homeostasis and its deficiency has a huge impact on bone mineral density, manifesting as rickets in children. Vitamin D deficiency commonly occurs in autoimmune diseases such as inflammatory bowel disease. It is, thus, expected that vitamin D supplement hinders the progression of these diseases. The active form of vitamin D can suppress inflammation in the IL-10-knockout mouse by inhibiting TNF-α pathway [141]. In a randomized double-blind controlled trial, Zerofsky et al. investigated the effect of daily cholecalciferol (vitamin D) supplementation, 400 IU/day vs. 2000 IU/day, during gestation on immunoregulatory markers in 57 pregnant women [142]. The supplementation was initiated at less than 20 weeks of gestation. The 2000 IU/day of vitamin D group had greater increase in the levels of serum 25-hydroxy vitamin D (81.1 nmol/L to 116 nmol/L) when compared to the 4000 IU/day group (69.6 nmol/L to 85.6 nmol/L) at 36 weeks of gestation ($P < 0.0001$). Moreover, the 2000 IU/day group exhibited more IL-10^+ $CD4^+$ T_{REG} cells ($P < 0.007$), which play a key role in regulating inflammation-related conditions in pregnancy [142].

In children, vitamin D deficiency is associated with increased risk of infections, including *Mycobacterium tuberculosis* infection and acute lower respiratory tract infections [143]. In another study, Zerofsky et al. found that asthma

diagnosis was significantly higher among children with rickets [144], confirming with other studies that reported maternal vitamin D deficiency as a predisposing factor for asthma development in children [145]. This might be due to the function of vitamin D in both the innate and adaptive immune response. It is necessary for cathelicidin production, an antimicrobial peptide that plays a role in mucosal immunity in the lungs [146]. It can also upregulate IL-10 expression – a regulatory cytokine – and favor the shift towards T_H2 cell-type response.

Moreover, vitamin D promotes the development of T_{REG} cells which modulate the immune responses by other subsets of T lymphocytes [147]. Type I diabetes mellitus is a T cell-mediated autoimmune disease characterized by destruction of β pancreatic cells, which secrete insulin. It is the outcome of an interaction between genetic and environmental factors. It is preceded by a preclinical stage, known as islet autoimmunity, characterized by the presence of autoantibodies against islet autoantigens. Much of the studies done focused on the association of vitamin D status and type 1 diabetes mellitus. The Diabetes Autoimmunity Study in the Young (DAISY) recruited children at birth and followed them for an average of 4 years [148]. The study showed that maternal vitamin D intake from food, rather than supplementation, was associated with reduced risk of islet autoimmunity independent of other potential risk factors (HLA genotype, family history of type I diabetes mellitus, presence of gestational diabetes mellitus, and ethnicity), with adjust hazard ratio of 0.37 (95% confidence interval of 0.17–0.78) [149]. This indicated that vitamin D from food had a protective role against the development of islet autoimmunity in the fetal stage.

Vitamin E

Vitamin E (α-tocopherol) functions as an antioxidant and plays a role in preventing or stalling the progression of free-radical-mediated diseases, such as CVD and malignancies [150]. It also influences the development of the immune system during the fetal and neonatal stages [151] and helps to maintain it healthy during adulthood [152]. Vitamin E is transferred to the progeny across the placenta during the fetal stage, via LPL action and very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) receptors [153], and through the milk during the neonatal period in a tight regulation. The infant, as in the case of vitamin A, exhibits low vitamin E stores at birth, regardless of maternal dietary supplementation. This might be due to the inefficient transfer of vitamin E across the placenta [154]. Therefore, vitamin E intake in milk is important, with higher content in colostrum than in mature milk [155, 156].

Vitamin E promotes monocyte/macrophage-mediated response. Hypovitaminosis E has been associated with impaired immune response and may enhance the virulence of viral infections [157]. Vitamin E decreases free radicals and impairs the formation of arachidonic acid metabolites, which, otherwise, result in suppressed immune response [130]. Studies have shown that vitamin E is beneficial in ameliorating the symptoms of eczema and atopic dermatitis and reducing the levels of IgE in subjects with these diseases [158].

Vitamin C

Vitamin C is an important antioxidant. Low concentrations of this vitamin are observed during infection and stress. Vitamin C supplementation has been proven to be beneficial for immunity against group A Streptococcal infections [159], by increasing serum levels of glutathione and thereby improving phagocytosis and NK cell function [160]. Vitamin C shows an antimicrobial effect against *Helicobacter pylori* [161] and its deficiency is associated with this bacterial infection.

B Vitamins

Maternal daily supplementation of 4–10 mg/day of pyridoxine (vitamin B5) during pregnancy increases IgG in cord and IgA, lysozyme, and lactoferrin in colostrum [162]. Maternal cobalamin (vitamin B12) deficiency is associated with histological impairment in the small intestine and depressed IgA-producing cells [163]. Maternal folate (vitamin B9) deficiency is associated with a skewed T_H1 response and allergic hypersensitivity [164]. In contrast, excess maternal folate intake during pregnancy is associated with higher

susceptibility to lower respiratory tract infections and asthma in children [165, 166], but this was not confirmed by meta-analysis [167].

Minerals

Iron

Prenatal stress results in suppressed NK cell function and iron-deficiency anemia [168]. The immune response is impaired in iron deficiency characterized by suppressed phagocytic activity and humoral response [169], increased pro-inflammatory cytokine response [170], decreased neutrophil function, and impaired T cell proliferative response [171]. One of the mechanisms used by the body to defend itself against pathogens is through iron sequestering. However, pathogens have evolved mechanisms to scavenge for iron even in the most deficient environments [78]. Therefore, because of its possible disadvantages, iron supplementation has been at the center of controversy due to "altered iron handling" [172]. Moreover, mild iron deficiency might be beneficial against malaria [173].

Zinc

Maternal zinc deficiency has been associated with poor fetal growth [174, 175]. Zinc plays a role in determining the timing, coordination, and progression of delivery through estrogen-dependent gene expression [176]. Zinc supplementation has been associated with increased birth weight as a result of lengthening gestation period and thereby reducing preterm delivery by 80%, particularly those occurring earlier than the 32nd week of gestation [177]. Prenatally, zinc maintains immune homeostasis, the integrity of cell membranes, synthesis of prostaglandins (PG), and central nervous system (CNS) development. Perinatally, it is vital for the transfer of immunological factors and vitamin A to the infant – maternal zinc deficiency is associated with a low maternal transfer of retinol [178]. Zinc deficiency would adversely affect the normal development of the fetal organs and cause immunodeficiency. A mild maternal zinc deficiency suppresses the fetal immune response [179], which is evidenced with thymic and spleen atrophy, impaired proliferation of lymphocytes, downregulation of both, T_H1 and T_H2 cell responses [180], and reduced concentrations of immunoglobulins [181, 182], in particular IgM and IgA, which might persist even after restoring normal zinc status [183]. A moderate maternal zinc deficiency, particularly prepregnancy, induces long-term deleterious effects on the fetal immune system. It suppresses primary and secondary humoral responses and causes persistent cellular immune deficiency [184]. In a randomized double-blind controlled trial, the effect of maternal supplementation with β-carotene (4.5 mg per day) and/or zinc (30 mg zinc sulfate per day), in addition to iron (30 mg ferrous fumarate per day) and folic acid (0.4 mg per day), on the morbidity and immune function of newborn infants was studied in pregnant Indonesian women with a gestational age of less than 20 weeks (n = 136) [185]. The infants were followed up for 6 months. When compared to control, zinc supplementation significantly reduced diarrheal episodes in infants (0.2 vs. 0.4 episodes, P = 0.032), but increased cough episodes (1.3 vs. 0.9 episodes, P = 0.028). Also, ex-vivo cytokine analysis showed an influence of maternal zinc and β-carotene supplementation. Zinc was successful in increasing the production of IL-6 and lowering IFN-γ production. Wieringa et al. concluded that the addition of zinc to routine maternal supplementation could be effective in reducing diarrheal episodes during the first 6 months of life [185]. However, in vulnerable populations, zinc deficiency or excess might lead to suppressed cellular immunity [186]. Zinc supplementation for restoring maternal zinc status (mild zinc deficiency) can improve mucosal cellular immune function. In contrast, maternal zinc supplementation in case of zinc adequacy prevents suppression of T lymphocyte function. This might be due to the sensitivity of T lymphocytes to intracellular zinc levels [187], zinc-induced copper deficiency [188, 189], or imbalance in retinol homeostasis [190]. Moreover, prenatal supplementation in zinc-adequate rats resulted in the suppression of antigen-specific proliferation, humoral responses, and antigen-presenting cell function in the offspring, while postnatal zinc

supplementation adversely affected mucosal immunity of the offspring [191].

Mycobacterium bovis BCG vaccine has long been used for the prevention of tuberculosis [192]. However, its efficacy varies from population to population (0–80%) [193]. Of note, zinc deficiency commonly occurs in regions with a high incidence of tuberculosis [194]. Therefore, Shi et al. investigated the effect of dietary zinc deficiency on the efficacy of BCG vaccine in offspring and adult rats [195]. Zinc deficiency enfeebled cell-mediated response to the vaccine by decreasing serum levels of IFN-γ and TNF-α, mRNA expression of ZIP2, ZIP8, NF-κB, and IL-6, and T cell proliferation [195].

Selenium

Selenium is a cofactor for the antioxidant enzyme, glutathione peroxidase. It suppresses the expression of manganese superoxide dismutase and uncoupling protein 2 [196]. It contributes to the function of leukocytes and NK cells and affects TLR signaling by repressing NF-κB activation. Selenium deficiency can be the result of PEM. Coexisting selenium deficiency and vitamin E deficiency can lead to increased virulence of coxsackievirus and influenza virus [197]. Selenium supplementation was effective in protecting against cell death caused by some viruses; it could increase survival in children diagnosed with HIV infection [198, 199]. In contrast, maternal selenium supplementation appears to promote HIV viral shedding and facilitated vertical transmission to infant [137].

Amino Acids

Tryptophan is an essential amino acid playing a vital role in the regulation of the immune system during pregnancy. Particularly, it has been proven beneficial against spontaneous abortion. An experimental study by Qiu et al. on the effect of tryptophan supplementation on pregnancy outcome in mice challenged with pseudorabies virus (PRV) suggested several mechanisms through which tryptophan may act. Increased dietary intake of tryptophan can help to improve pregnancy success, by maintaining synthesis of circulating immunoglobulins such as IgM and IgG; providing a substrate for indoleamine 2,3-dioxygenase (IDO), whose catabolites act directly to induce apoptosis in T_H1 cells, but not in T_H2 cells [200];, blocking PRV-induced production of T_H1 cell cytokines (such as IFN-γ and IL-2) and PRV-induced inhibition of T_H2 cell cytokines (such as IL-10) [201]; and increasing progesterone in the placenta, which, in turn, would significantly suppress T_H1 cell development and induce the production of IL-10-producing cells [202]. The effect of tryptophan supplementation on IgG is interesting since this is the sole immunoglobulin that can cross the placenta, thereby protecting the embryo against the invading pathogens. In this manner, tryptophan would act to shift the balance of T_H1/T_H2 cytokines toward T_H2, thereby providing support for pregnancy [201].

Omega-3 and Omega-6 Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) have been extensively related to neurological and eye development. PUFAs also play a role in immune cell functioning, through maintaining membrane fluidity and regulating gene expression, signal transduction, and cellular function [203]. There are two PUFAs superfamilies, ω-3 PUFAs and ω-6 PUFAs, which are believed to act antagonistically to control physiological functions [204]. Dietary linoleic acid (C18:2 ω-6) is abundant in many vegetable oils (corn, sunflower, soybean) and is converted into long-chain polyunsaturated fatty acid (LCPUFA) in the body, such as arachidonic acid (C20:4 ω-6). Arachidonic acid generates, by the catalytic action of lipoxygenase (LOX) or cyclooxygenase (COX), a number of eicosanoids such as PG, thromboxanes (TX), and leukotrienes (LT). These, in turn, regulate the intensity and the duration of inflammatory responses and play a role in promoting sensitization to allergens. Dietary α-linolenic acid (C18:3 ω-3) is abundant in green plants, some vegetable oils (soybean, rapeseed), and some nuts and seeds and is converted into ω-3 LCPUFA, eicosapentaenoic acid (EPA) (C20:5 ω-3) and docosahexaenoic acid (DHA) (C22:6 ω-3). Of note, oily fish such as

mackerel and salmon are considered as rich sources of pure EPA and DHA, which are known for their anti-inflammatory effects. Studies link increasing dietary intake of EPA and DHA to a reduced production of arachidonic acid-derived eicosanoids and therefore decreased the intensity of inflammation [205]. On one hand, EPA competes with arachidonic acid for binding to the enzymes that produce inflammatory eicosanoids. On the other hand, EPA and DHA, respectively, produce E-series resolvins and D-series resolvins, which have anti-inflammatory actions [206]. Therefore, targeting PUFAs and using ω-3 fatty acids might be therapeutic for inflammatory conditions, including allergic diseases [207–210].

During the fetal period, immune responses against antigens are launched as early as the 25th week of gestation [211]. Therefore, this period of development is vital in determining future immunological and clinical outcomes [212]. Allergic children are characterized by sustained skewness towards T_H2 cell response and elevated IgE production [211]. Observational studies have shown that allergic sensitization and disease risk can be reduced when dietary ω-3 PUFA is consumed as part of the diet during pregnancy [213, 214] or during early childhood [215, 216]. Overall, epidemiological studies show that fish consumption during infancy or childhood has positive effects outcomes to reduce the risk of atopic disorders by 22–80% [203]. In a randomized placebo-controlled trial, Furuhjelm et al. examined the effect of daily fish oil supplementation, 1.6 g of EPA and 1.1 g of DHA, during gestation and lactation, on allergic disease risk in infants. The supplementation was given to mothers, who themselves were affected by allergy or had a husband or previous child with an allergy. It was initiated at the 25th week of gestation and continued until 3–4 months after delivery [217]. Fish oil supplementation could effectively decrease the risk of food allergy and IgE-associated eczema during the first year of life in infants [217]. The *Australian Fish Oil Supplementation Study* showed that maternal fish oil supplementation resulted in higher EPA and DHA status, lower arachidonic acid status [218–220], lower oxidative stress [221], lower cord plasma IL-13 concentrations [222], lower cord blood mononuclear cell cytokine response to allergens [222], lower LTB4 production, and higher LTB5 production by cord blood neutrophils [223]. The *European Multicenter Pregnancy Supplementation Study* found that maternal fish oil supplementation downregulated maternal T_H1 responses while upregulating fetal T_H2 responses, where there were lower mRNA expression of IL-4, IL-13, and chemokine receptor (CCR)-4, reduced frequency of NK cells and $CCR3^+$ $CD8^+$ T cells in cord blood, and reduced mRNA expression of IL-1 and IFN-γ in maternal blood [224]. Moreover, the long-term effect of maternal fish oil supplementation was observed in the Danish Follow-up Study. After 16 years, the odds ratios for asthma and allergic asthma were decreased by 63% and 87% in the offspring of the mothers who consumed fish oil supplementation during pregnancy [225]. The *Copenhagen Prospective Study of Asthma in Childhood birth* cohort involved 411 children of mothers with asthma and the effect of maternal fatty acid desaturase (*FADS*) genotype and breast milk long-chain PUFA levels on T cell profile and cytokine levels of 109 infants was assessed [226]. Muc et al. observed an inverse correlation between arachidonic acid content in milk and the production of IL-10 (r − 0·25; P = 0·004), IL-17 (r − 0·24; P = 0·005), IL-15 (r − 0·21; P = 0·014), and IL-13 (r − 0·17; P = 0·047), while EPA showed positive correlation with T_{REG} and cytotoxic T cell count. Maternal long-chain PUFA synthesis and breast milk arachidonic acid are correlated with depressed expression of IL-5, IL-13, IL-17, and IL-10 in the infant [226].

However, dietary supplementation of these fatty acids (5 g fish oil or safflower oil/kg per day) in neonatal rabbits with staphylococcal pulmonary infection – which is the most common cause of nosocomial pneumonia in intensive care nurseries – showed adverse effects in terms of reduced pulmonary clearance of inspired Staphylococcus aureus and lowered macrophage superoxide anion generation [227].

The Environmental Determinants of Diabetes in the Young (TEDDY) study recruited 424,788 newborn infants for a longitudinal prospective observational study of type I diabetes mellitus and celiac disease. The study found no associa-

tion between maternal use of supplements (vitamin D, ω-3 polyunsaturated fatty acids, or iron) and risk of celiac disease in the child [228].

Food and Food Groups

High-Fat Diet

Maternal diet during pregnancy and lactation affects the development and function of the immune system of the offspring. A high-fat dietary environment in utero leads to obesity, immunological disorders, and nonalcoholic fatty liver disease because of imbalance in adipokines associated with obesity. Odaka et al. investigated the influence of maternal high-fat diet during gestation on the postnatal metabolic and immune function in mice. Sixteen pregnant female mice received control diet and 16 pregnant female mice received high-fat diet. Following parturition, lactating mice were given either control or high-fat diet, leading to four groups. The results showed that maternal high-fat diet increased fat mass and serum glucose, leptin, and triglyceride levels. Leptin is an adipocytokine that influences immune function by induction of T_H1-type cytokine production and adhesion molecule expression [229, 230]. However, obese correlates with leptin resistance, meaning that leptin would not be able to exert its immunomodulatory function in obese individuals despite hyperleptinemia [229, 231]. The fetal high-fat dietary environment would impair immune homeostasis as reflected in a reduced number of splenic lymphocytes, thinner thymic cortex, altered antigen-specific antibodies, and elevated levels of TNF-α. Furthermore, the group feeding high-fat diet throughout pregnancy and lactation exhibited consistent depression in ovalbumin-specific IgG levels and elevated IgE levels, which constitute fatty liver changes. Overall, a high-fat dietary environment cannot only cause obesity, but is capable of influencing the immune homeostasis in the offspring [232].

Dairy Products

Cow's milk allergy is the most common allergy, affecting approximately 2–6% of children [233]. A study showed that maternal consumption of dairy products during pregnancy was associated with lower risk of developing cow's milk allergy in infants (OR = 0.56) and high IgA levels in cord blood, indicating a possible protective effect against the allergy, particularly in those of nonallergic mothers [233].

Pre-germinated Brown Rice

Regular brown rice is soaked in water to slightly germinate forming pre-germinated brown rice, according to the patented method (JP3738025). Studies have shown beneficial effects of pre-germinated brown rice due to its high fiber content. More precisely, it can decrease postprandial blood glucose in diabetes mellitus [234, 235] and control cancer cell proliferation [236] and neurodegeneration [237]. In the study [238], the effect of pre-germinated brown rice on mental maternal health and immunity was investigated in 41 lactating mothers. Compared with the control group, the group who received pre-germinated rice scored less on the profile of mood states test (POMS). Particularly, they reported reduced depression, anger-hostility, and fatigue. This might be due to the fact that pre-germinated brown rice contains higher amounts of γ-aminobutyric acid (GABA) (150 mg/kg vs. 20 mg/kg) and vitamin B1 (4.2 mg/kg vs. 0.8 mg/kg) than white rice [238]. Also, breastfeeding mothers reported lower stress levels as measured by salivary amylase activity, a rapid and sensitive marker of stress [239], than non-breastfeeding mothers [240]. This can be explained by the suppressive effect lactation has on the HPA axis [241]. Stress levels were further decreased in the pre-germinated brown rice group in comparison to white rice group. In this manner, the CNS and the immune system interact with one another and nutrient factors seem likely to mediate this interaction. Under psychosocial stress, secretory IgA in breast milk is reduced [242]. The pre-germinated brown rice diet was effective in increasing secretory IgA and lactoferrin content of breast milk [238].

Vegetables

The DAISY birth cohort showed that increased potato consumption in late gestation is inversely associated with the risk of developing islet autoim-

munity in infants [243]. In-utero exposure to certain dietary components, such as bafilomycin in potatoes and root vegetables [244], gluten [245], or glycemic effect of carbohydrates, might play a role in determining the risk for developing islet autoimmunity. All Babies in South-east Sweden (ABIS) study involved 21,700 infants, 16,004 of whom completed screening questionnaires after delivery [246]. The children were followed at intervals 1, 2.5, and 5 years of age, and 5724 of those were tested for islet autoimmunity. The study found that lower daily consumption of vegetables (3–5 times per week) is associated with increased risk of developing the disease (Or = 1.71; 95% CI, 1.24–2.35, P = 0.001). Brekke and Ludvigsson concluded that increased maternal intake of vegetables during pregnancy is associated with reduced risk of islet autoimmunity in infants. Interestingly, this seemed to be due to factors other than antioxidant content (vitamin C & E) of vegetables, since fruits are a richer source of antioxidants than vegetables, but were not effective as much as vegetables for the prevention of islet autoimmunity [246].

Maternal Nutrition and Neonatal Microbiota

Passive immunity is the process by which the neonate acquires innate and adaptive immunity through the mother, either through the placenta during the fetal stage or breast milk perinatally [247]. Early breastfeeding bolsters the formation of optimal microflora in the infant, such as *Lactobacillus rhamnosus* [5]. Secretory IgA, present in breast milk, plays a role in determining the composition of commensal bacteria in the small and large intestines and therefore the development of the microflora and evolution [248, 249]. There are more than 400 strains of bacteria residing in the small and large bowel [250], which in addition to food digestion [251] aid in protecting against pathogens [252]. The microflora interacts with TLRs in the intestinal mucosa to regulate immune response toward T_H1- or T_H2-type response [253]. *Lactobacillus* species play a role in mucosal tolerance by priming DCs to induce the development of T_{REG} cells [254]. Diaz et al. investigated the influence of maternal immune system on the pattern and abundance of intestinal microflora of mice fostered by wild-type (WT) or recombination-activating (RAG) gene knockouts (B and T cells deficient) dams [255]. The study found that *Bacteroides* species were the most influenced by the maternal immune system status; with more than a hundred-fold increase in WT dams than RAG-knockout dams. This suggests that the maternal adaptive immune system induced the expansion of *Bacteroides* colony in the large intestine. In general, bacterial densities were significantly lower in the small intestine of mice fostered by WT dams, but were more abundant in the large intestine, indicating that the maternal adaptive immune system regulated the microflora in an age- and site-specific manner [255]. Studies have shown that the immune system of breastfed infants cultivated probiotic organisms [256, 257], such as *Lactobacillus* and bifidobacteria, under the influence of secretory IgA that prevents the growth of pathogenic bacteria and milk oligosaccharides that act as prebiotics [258, 259]. Maternal probiotic supplementation around birth altered the composition of gut microflora and the immunomodulatory effects of milk on the offspring [253].

Maternal and Neonatal Stress, and Immunity

Maternal psychological stress negatively affects the ontogeny of the fetal and neonatal immune system, by inhibiting macrophage and neutrophil function and reducing cytotoxicity of NK cells during the third trimester [260]. Animal studies showed that maternal stress would influence the size and morphology of thymus in neonates [261]. It has been shown to impair thymus function and reduce the number of total lymphocytes [262], $CD4^+$ T cells, and $CD8^+$ T cells in adulthood [263]. It also age-specifically diminishes T lymphocyte functions (mitogenicity and delayed-type hypersensitivity) [80] and humoral immunity (antibody production) [80]. Moreover, maternal stress affects the transfer of passive immunity to the fetus across the placenta, with a reduction in fetal IgG levels at birth in rats [264]. This result was not consistent in

all animal studies. For example, there was no effect of maternal stress on IgG levels in offspring of mice [265], and a gender-specific effect was observed in squirrel monkeys [266].

Animal studies investigating the effect of stressful events during gestation and infancy showed impairment in mucosal immunity and microflora composition in the infant's gut. Kang et al. investigated the effect of maternal depressive symptoms during and after gestation on fecal secretory IgA levels in 403 term infants [267]. The results showed that infants born to mothers with pre- and postnatal depressive symptoms had significantly lower levels of fecal secretory IgA when compared to control infants (4.4 mg/g feces vs. 6.3 mg/g feces; $P = 0.033$). This might make these infants more susceptible to allergic diseases [267].

Neonatal stress impairs the immune system in adulthood, regardless of HPA reactivity. Mild neonatal stress suppresses T_H2-type response while activating T_H1 cell-type response [268]. It is associated with gastrointestinal diseases, such as Crohn's disease [269] and irritable bowel syndrome [270], and increases susceptibility to pathogenic bacteria. Barreau et al. studied the effect of maternal deprivation on the colonic epithelial barrier function and mucosal immunity in neonatal rats. The separated neonates exhibited elevated colonic paracellular permeability, as evidenced by macroscopic damage, increased colonic myeloperoxidase activity, higher mucosal mast cell density, and increased mRNA expression of several cytokines, including IL-1β, IL-2, IL-4, IL-10, and IFN-γ [271]. This would result in an increased risk of bacterial translocation to mesenteric lymph nodes, liver, and spleen. The maternally deprived neonates also exhibited an exaggerated immune response to 2,4,6-trinitrobenzene sulfonic acid (TNBS), as an external stimulus, indicating their increased susceptibility to colonic inflammation and/or hypersensitivity on the long term [271]. Maternal deprivation also adversely affected macrophage activity and increased susceptibility to experimental autoimmune encephalomyelitis in rats [272]. Barreau et al. also examined the effect of maternal deprivation on parasitic infection by *Nippostrongylus brasiliensis* on adult rats. The authors discovered that it has accelerated the primary infection by the parasite as evidenced by a 50% increase in the number of parasites. However, immunity breakdown was not severe upon secondary infection [273].

Maternal Physical Activity and Fetal Immunity

Regular physical activity promotes an anti-inflammatory state. It is thus encouraged before and during pregnancy as an important strategy in defying adverse effects of maternal stress and malnutrition on the fetus. Physiologically, metabolic and psychological effects of physical activity help to reduce morbidity and mortality in the mother and her child [70]. Physical activity can reduce prenatal stress, which has adverse effects on the infant cognition, HPA axis function, and neurogenesis. It reduces the risk of hyperglycemia, hyperbilirubinemia, macrosomia, and fetal complications associated with preeclampsia such as intrauterine growth restriction. Physical activity, even acutely, can induce an anti-inflammatory state, promote endothelial function by increasing nitric oxide bioavailability, and enhance vasodilation by suppressing TNF-α level [70].

Conclusions

Maternal nutritional status, maternal stress, and microbiota have been proven to be among factors that determine the ontogeny of the immune system during the fetal and neonatal periods. They are capable of influencing both humoral and cellular immunity and therefore the future risk of developing diseases, such as inflammatory and autoimmune diseases, allergy and atopic diseases, and cardiometabolic diseases. By manipulating nutritional status and maternal stress, it might be possible to hinder the progression of these high-burden diseases. Further investigations are needed to determine the cutoff points, particularly concerning maternal macro- and micronutrient deficiencies.

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Nutrition, Immunity, and Cancer

12

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Key Points

- Under special circumstances and defects in resolution process or if its underlying factors continue, then inflammation will turn into chronic inflammation.
- Chronic inflammation can increase the risk of cancer through promoting tumor initiation, the rate, and extent of cell division, neovascularization, and angiogenesis.
- Chronic inflammation results in an overload of reactive oxygen species (ROS), which, in turn, may lead to the development and progression of cancer.
- Immune escape mechanisms are a hallmark of tumor progression.
- Bioactive dietary components that antagonize immune escape mechanisms would have potential to prevent tumor development or enhance tumor regression.

Dietary Components, Immunity, and Cancer

Acetylsalicylic acid is a nonsteroidal anti-inflammatory drug (NSAID) that has shown chemopreventive effects in animal models and to

reduce both inflammation and cancer risk in humans [1]. Salicylic acids exist in a wide range of fruits, vegetables, herbs, and spices. It has been shown that regular intake of salicylates may be causally associated with reduced incidence of certain cancers, especially colon cancer [2].

Macronutrients and Immune System Modulation

Amino Acids

Arginine and glutamine are depleted during the immune response. Arginine is a precursor of polyamine, which is necessary for fidelity of DNA transcription. In addition, arginine is the only substrate for iNOS. Because of reduced arginine concentrations in plasma, T-cells are downregulated by the accumulation of myeloid-derived suppressor cells (MDSCs) and arginase-1 secretion. Glutamine plays a role to sustain lymphocyte proliferation, increase phagocytosis by onocytes/macrophages, and enhance neutrophil cytotoxicity [3]. On the other hand, sulfur amino acids are essential for the generation of glutathione, acting against prooxidant effects of inflammation and aiding cytotoxic T (T_C)-cell activation [4].Tryptophan is another important anti-inflammatory molecule, which is found in various types of vegetables and fish. Tryptophan is converted into indole-3-aldehyde, the ligand of aryl hydrocarbon receptor (AhR), by bacterial enzymes (e.g., lactobacilli). AhR functions as a receptor for dietary components and as a transcription factor expressed in epithelial and immune cells and some tumor cells. Several phytochemicals and plants from the Brassicaceae family have been shown to influence AhR ligands. Anti-inflammatory effects of tryptophan can occur through conversion of indoleamine-2,3-dioxygenase to kynurenine. Both indoleamine-2,3-dioxygenase (IDO) and kynurenine modulate T-cell function. Moreover, kynurenine which is produced by cancer cells can suppress antitumor immune responses [5, 6]. AhR can mediate the effects of diet to produce anti-inflammatory effects by affecting microbiota and gut immunity.

Lipids

Increasing the ratio of n-3 to n-6 polyunsaturated fatty acids (PUFA) (n-3/n-6) is generally in favor of human health. High n-3/n-6 ratio has been associated with increased anti-inflammatory responses and decreased risk of cancer. Inflammatory cells display high proportions of n-6 PUFA and low proportions of n-3 PUFA; thus, enhancing the dietary intake of n-3 PUFA could affect the amount and type of endogenously produced eicosanoids [7]. High intake of n-3 PUFA causes replacement of arachidonic acid (AA) in inflammatory cell membranes by eicosapentaenoic acid (EPA) and decreased generation of AA-derived mediators that regulate the secretion of cytokines. Other possible effects may occur through modification of membrane fluidity and lipid rafts and also changes in the gene expression and antigen production associated with signal transduction [8]. For instance, a highly purified form of n-3 PUFA, docosahexaenoic acid (DHA), not only altered the composition of T-cell membrane but also downregulated signaling pathways of activator protein-1 (AP-1), NF-κB, and IL-2 and lymphoproliferation. Also, it has been reported that omega-3 can decrease the expression of pro-inflammatory adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin [9]. Short-chain fatty acids (SCFAs), e.g., acetate, butyrate, and propionate, which are produced by colonic bacteria appear beneficial for regulatory T (T_{reg})-cell proliferation [10, 11]. Phase III clinical trials have been published confirming the efficacy of omega-3 supplementation in some types of cancer.

Minerals

Trace elements, in particular, zinc, iron, and selenium, play a key role in the regulation of immune responses [12]. Zinc deficiency can cause a shift from T_H1 to T_H2 immune responses, result in the activation of macrophages and monocytes, and increase the production of pro-inflammatory cytokines (tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, and IL-8) [13, 14]. Selenium has been most strongly associated with cancer risk [15]. Selenium not only does act as an antioxidant by participating in the structure of

glutathione peroxidase but also can decrease the sensitivity of lymphocytes to oxidative stress (OS). Its deficiency decreases neutrophil chemotactic activity and antibody generation by B-cells. By contrast, supplementation with selenium would increase phagocytosis, NK cell activity, and T-cell responses [16].

Vitamins

Retinoic acid, the active metabolite of vitamin A, contributes to the activation of nuclear factor receptors-α (RARα), RARβ, and RARγ, which are essential for the stability of T_H1 cells and for controlling conversion from T_H1 cells to T_H17 cells. Antioxidant vitamins like vitamins C and E are able to scavenge free radicals [17]. Vitamin B6 significantly affects the expression of iNOS and COX-2 induced by lipopolysaccharide (LPS). This vitamin inhibits the induction of NF-κB by LPS and leads to a reduction of LPS-induced I-B degradation in RAW cells. Vitamin D and calcium deficiencies interfere with cellular functions in multiple tissues and organs, including the immune system [18]. Betaine (trimethylglycine) is a vitamin-like substance that acts as a methyl donor. Study of aged Sprague Dawley (SD) rats showed that this nutrient has the ability to reduce renal expression of genes encoding inflammatory mediators such as NF-κB, COX-2, iNOS, VCAM-1, and ICAM-1 [19].

The relation of vitamin D3 to immune function and cancer has been the subject of numerous studies. Besides immune cells (macrophages, monocytes, dendritic cells (DCs), and dermal cells), the 25-hydroxyvitamin D3 is metabolized to 1,25-dihydroxyvitamin D3 in the kidneys. Genes that show differential expression in response to vitamin D include nuclear factor of activated T-cells (NFAT), nuclear factor of activated B-cells (NFAB), epidermal growth factor receptor (EGFR), c-myc, and keratin (K16). Vitamin D as an alternative to classical immunosuppressive agents is used in secondary malignancies. Vitamin D supplement has been beneficial for patients with prostate, breast, and colorectal cancer (CRC) and melanoma. Studies support its potential as an adjuvant for cancer [20, 21]. Vitamin D supplement improved disease-free survival in patients with early-diagnosed breast cancer and metastatic CRC. There was a positive association between disease-free survival and plasma 25-(OH) D3 levels [22].

Vitamin E improves immune function through its antioxidant property. Antioxidant parameters including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) enzyme family, and vitamins C and E have the potential to serve as biomarkers of prostate cancer [23]. Daily intake of greater than 100 IU vitamin E has been demonstrated to reverse T-cell function impaired by senescence [24]. Additionally, a Bayesian meta-analysis has clearly proved the safety of vitamin E [25]. In vitamin C deficiency, phagocytic activity is impaired because of low neutrophil numbers and reduced NK cell functions [26]. Results from a meta-analysis point to the benefit of decreased mortality that patients diagnosed with breast cancer obtain from vitamin C supplement.

Dietary Bioactive Compounds and Cancer Prevention Through γδ-T-Cells

About 30% of all malignancies in the Western world are estimated to be diet related, where overconsumption of definite food items or not enough of others in whole diet may contribute to cancer incidence [27]. Thus, cancer appears to be potentially preventable or modifiable by suitable dietary interventions. For example, fruit and vegetable consumption reduces the risk of bladder [28, 29] and gastric cancer [30, 31]. Also, reduced risk of prostate cancer has been reported to be in association with cruciferous vegetable consumption and high vitamin C intake [32, 33]. Dietary components can modify the risk of cancer by affecting various processes, including DNA repair, differentiation, apoptosis, angiogenesis, and modification of immune responses. As suppression of immunity is associated with increased risk of cancer, maintenance of immune homeostasis may have the potential to decrease cancer risk [34]. This part will address **γδ**-T-cells, their ability against malignant cells, and diet-mediated changes in **γδ**-T-cell function. Several in vivo and in vitro studies reported that certain food

components might modify **γδ**-T-cell differentiation and function. We will discuss the possible effect of dietary bioactive compounds in preventing cancer through **γδ**-T-cell-based mechanisms.

Based on the T-cell receptor (TCR) on their surface, there are two main subgroups of T-cells: αβ-T-cells that account for about 95% of T-cells in peripheral blood and γδ-T-cells that account for 0.5–5% of all T-lymphocytes [35, 36]. αβ-T-cells commonly express CD4 or CD8 lineage markers [35]. **αβ**-T-lymphocytes mostly belong to helper or cytotoxic/effector subsets [37, 38]. On the contrary, γδ-T-cells do not generally express CD4 or CD8 lineage markers. T-lymphocytes usually recognize antigenic peptides by major histocompatibility complex (MHC). However, γδ-T-cells do neither require conventional MHC antigen presentation [35] nor recognize peptide antigens on antigen-presenting cell (APC) surfaces. In fact, these cells are activated in the way similar to that of the innate immune cells, meaning through recognition of pathogen-associated molecular patterns (PAMPs) [39, 40], damaged tissue [41, 42], and targets of NK-associated receptors [43, 44]. Phosphorylated uridine and thymidine compounds [45], non-protein prenyl pyrophosphates [46, 47], bisphosphonates [47, 48], and alkylamines [49, 50] have all been reported to activate or prime γδ-T-lymphocytes. Alkylamines can be obtained from the diet and include compounds such as ethylamine, butylamine, and propylamine. Other PAMPs include heat shock proteins [51] and intermediates from the mevalonate pathway which is induced in response to self's distress signals [52]. The mevalonate pathway is common to all cells, particularly malignant cells, which can be influenced by several dietary factors such as cholesterol, isoprenoids, and genistein [53].

There are two main subsets of γδ-T-cells in mammalian species: Vδ2-T-cells which are mainly found in circulation and Vδ1-T-cells which are specific to mucosal surfaces lining the respiratory, gastrointestinal, urinary, and reproductive tracts [54]. Circulating γδ-T-cells produce effector functions against invading pathogens and malignant cells and could migrate to sites of infection [55]. The mucosal population assists in the maintenance of epithelial barrier integrity through diminishing inflammatory responses and healing of the damaged tissue [56–58]. γδ-T-cells are on the frontline to respond to invading pathogens and pave the way for the rest of the immune cells to participate in the elimination of invading pathogens.

γδ-T-cells share features of both innate and adaptive immune cells [59]. These cells produce high amounts of cytokines, chemokines, and growth factors. In this respect, the most important cytokine is interferon (IFN)-γ which is involved in antitumor immune responses [60]. In addition, γδ-T-cells support humoral immunity by the production of IgA, IgM, and IgG antibodies [61]. Other important roles include recruiting macrophages and inducing cytotoxicity in malignancies by producing a variety of chemokines like perforin-granzyme and TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor (TRAILR) system [62].

γδ-T-Cells in Cancer

γδ-T-cells can directly reject tumor cells through different ways. They have the ability to secrete cytokines such as IL-4, IL-10, TNF-α, and IFN-γ [61, 63, 64] which promote antitumor immunity. By increasing the expansion of $CD8^+$ T-cell, monocytes, and neutrophils and upregulating the expression of Fas ligand (FasL) and TRAIL, γδ-T-cells enhance tumor killing activity in the Fas- or TRAIL receptor-sensitive tumors [65, 66]. CD16 is a receptor for the Fc portion of immunoglobulin G (Fcγ receptors). γδ-T-cells by expression of CD16 can increase antibody-dependent cellular cytotoxicity (ADCC) [67]. In addition, γδ-T-cells elicit the release of granzymes and perforin that mediate cellular apoptosis [68] and interact with professional APCs that process antigens important for the killing of target cells [69]. Another function of the γδ-T-cells is the ability to moderate or end inflammation by inhibition of macrophage activation [70, 71]. Interestingly, antigens in bioactive dietary compounds that resemble PAMPs can prime γδ-T-cells, thereby attenuating inflammation and cell damage, which have been implicated in cancer.

Bioactive Dietary Compounds and Possible γδ-T-Cell Activity Against Cancer

The modified function of γδ-T-cells by dietary bioactive compounds may cause favorable immunological response. Information regarding the effect of dietary compounds on differentiation of γδ-T-cells is limited. Vitamins A and D have been reported to play a role in γδ-T-cell differentiation [72]. Vitamin D receptor on the surface of γδ-T-cells is upregulated via a protein kinase C (PKC)-related mechanism [73]. The relationship between diet and γδ-T-cells was first drawn in 1999 [74], when drinking tea increased γδ-T-cell proliferation and IFN-γ secretion compared with coffee. L-Theanine is a bioactive compound available in tea. L-Theanine is hydrolyzed to glutamic acid and ethylamine, a nonmicrobial antigen that interacts with γδ-T-cells [75, 76]. Two classes of plant metabolites have been characterized with defined effects on γδ-T-cells, including non-protein prenyl pyrophosphates [77] and procyanidins [78] that induce Vδ2-T- and Vδ1-T-cells, respectively. Many other bioactive compounds are being investigated.

The hypothesis of nonmicrobial priming implies that though food phytochemicals cannot activate cells, they can prime the cells to respond better and more rapidly to a secondary antigen [79]. Previous in vitro experiments indicated that proanthocyanidins interact with γδ-T-cells and increase their proliferation and activation [80].

In a previous study, consumption of fruit and vegetable concentrate increased γδ-T-cells in the blood while decreasing circulating IFN-γ concentrations [81]. In another study, a capsule containing a standardized mixture of tea components, L-theanine and catechins, was reported to influence γδ-T-cell function. People consumed a distinct amount of L-theanine and catechins for 10 weeks. White blood cells (WBC) from the participants were incubated ex vivo, with the compound responsible for priming, ethylamine. Greater activation and proliferation of γδ-T-cells and greater concentration of IFN-γ were observed in subjects consuming L-theanine compared with placebo. As a side note, subjects taking the capsule experienced fewer cold and flu symptoms during the study [82, 83].

Mistletoe has been reported to increase levels of IL-12 [84], a cytokine that supports the proliferation and cytotoxicity of γδ-T-cells [85]. In another study, mistletoe extracts (50–500 mg/L) increased proliferation of γδ-T-lymphocytes in vitro in a dose-dependent manner [86]. In two strains of mice, at first food allergy was established with ovalbumin sensitization; and then feeding apple condensed tannins (ACT) resulted in much less severe anaphylaxis, lower histamine levels, and decreased serum levels of IgE, IgG1, and IgG2a. γδ-T-lymphocytes were significantly increased in the intestinal epithelium of those consuming ACT [87]. In another in vitro experiment, a quite low concentration (20–40 mg/ml) of apple polyphenols upregulated CD11b on γδ-T-cells [88].

Dietary nucleotides have been indicated to change the percentage of intestinal intraepithelial γδ-T-cells [89]. Adding 0.4% nucleotides to the regular diets of weanling mice for 2 weeks increased γδ-T-cell proportion from 50.6% to 58.7% and increased secretion of IL-7, but not IL-2 or IFN-γ.

Different dietary oils have been investigated regarding their possible effect on γδ-T-cells. In one study, splenic γδ-T-cells were statistically higher in the safflower oil diet compared with the fish oil diet. The possible response to n-6/n-3 fatty acid ratio has been suggested [90]. Conjugated linoleic acid (CLA) has also been reported to almost double the number of γδ-T-cells in pigs fed 1.33 g CLA/100 g diet for 72 days [91]. Vaccination combined with CLA increased γδ-T-cell numbers largely (sixfold).

Alkylamine compounds produced by gut microbiota have been shown to prime γδ-T-cells [50, 92]. Furthermore, they can be obtained from dietary sources, such as kola nuts [93], tea, apple skins, mushrooms, and cucumbers [92]. Drinking tea increases urinary ethylamine [75]. When mixed with peripheral blood mononuclear cells (PBMCs), ethylamine could cause a 15-fold increase in the number of γδ-T-cells [92]. In addition, the secretion of IFN-γ in PBMCs incubated with ethylamine and challenged with bacteria was shown to be stimulated by alkylamines [50, 92]. Consumption of tea caused a two- to threefold increase in the capacity of γδ-T-cells to

secrete IFN-γ in response to bacterial pathogens or nonpeptide antigens.

A trial in healthy individuals showed that regular consumption of Concord grape juice for 9 weeks significantly increased the number of circulating γδ-T-cells [94]. Consumption of *Lentinula edodes* (shiitake) mushrooms for 4 weeks also led to an increase in ex vivo proliferation of γδ-T- and NK T-cells and in sIgA production [95]. Studies also investigated the effects of aged garlic extract (AGE) in healthy subjects [96] and patients with cancer [97]. Although not many γδ-T-cells were found in the serum, they were expanded in the epithelial linings of the gastrointestinal, respiratory, and genitourinary tracts [96]. A trial in healthy subjects revealed that the proliferation index of γδ-T-cells was almost five times increased after a 10-week cranberry juice consumption [98]. Other plant preparations with γδ-T-cell agonist activity include compounds from *Funtumia elastica* bark, *Angelica sinensis* root, cocoa, cat's claw bark, grape seed extract, and safflower oil [99–102]. Recent evidence reveled that grape seed extract has potent γδ-T-cell agonist activity. On the other hand, cocoa extracts caused expansion of rat γδ-T-cells in vivo [101] to some degree similar to that observed with apple-derived procyanidins [87]. Of note, the expansion of γδ-T-cell population particularly occurred in intestinal and Peyer's patches after oral administration of procyanidins. Rats feeding cocoa showed an increase in intestinal γδ-T-cells and a decrease in production of secretory IgA [87, 101].

In one study in mice, the effect of methanol extract from *Chelidonium majus* was investigated in collagen-induced arthritis. *Chelidonium majus* decreased B-cell and γδ-T-cell numbers (in spleen) while increasing the proportion of $CD4^+CD25^+$ T_{reg} cells [103]. The production of cytokines (TNF-α, IL-6, and IFN-γ) and the levels of IgG and IgM RA factors were decreased as well [103]. One experiment showed that condensed tannins derived from the unripe peel of the apple fruit act as agonist for both human Vδ1- and Vδ2-T-cells and increase the expression of IL-2R and cell proliferation. Previous studies reported that glutamine prevents apoptosis of small intestinal γδ-T-cells and downregulates the expression of inflammatory mediators by γδ-T-cells in septic mice [104, 105].

However, it has been discussed that many of the bioactive compounds in diet are only absorbed minimally, and their ability to influence immune responses throughout the body is therefore argued. However, it must be noted that several bioactive compounds do not need to be absorbed by the body to modify immune cells. For example, such compounds may be metabolized by the microbiota, and intermediates which are absorbed in the colon influence circulating immune cells. However, this has not been proven yet. Furthermore, Peyer's patches and intraepithelial cells lining the microvilli contain several immune cells, many of which express γδ-TCR. In addition, gut immune cells are able to move in and out of tissues via the circulation and the lymphatic system [106]. In this manner, blood-borne γδ-T-cells would be influenced by bioactive compounds which have not yet been absorbed.

Although tumoricidal activity of bioactive food compounds has not been clearly shown, certain food components are known to prime γδ-T-cells. When primed cells encounter a malignant cell, they can respond faster and more efficiently in terms of increased production of cytokines. However, enhancement of immune function is not always favorable; it is associated with decreased risk of cancer on one side, and on the other side, it has the potential to increase the risk of autoimmune diseases such as inflammatory bowel disease [107] and celiac disease [108]. Further research is necessary to investigate the relevance of using bioactive food components as regulators of γδ-T-cell function. If results support the hypothesis of priming γδ-T-cells, then this would propose a mechanism by which dietary factors can reduce the risk of cancer.

Cocoa, Immunity, and Cancer

Cocoa, the dried, roasted, and either unfermented or fermented seeds derived from *Theobroma cacao* tree, has been consumed by ancient civilizations such as the Mayans and Aztecs [109, 110]. Cocoa or cacao contains the highest flavanol

content of all foods on a weight basis and is a significant contributor to total dietary flavonoid intake [111]. It is worth mentioning that manufacturing processes increase flavonoid contents of cocoa four times greater than in conventional cocoa powder [112]. In this respect, fermented cocoa contains high quantities of flavonoids, flavanols (also called flavan-3-ols), (−)-epicatechin (EC), and (+)-catechin and to a lesser extent other polyphenols such as quercetin, naringenin, luteolin, and apigenin [113]. When compared to other flavonoid-containing foodstuffs, cocoa and its derivatives contain high concentrations of procyanidins, which are weakly absorbed through the gut barrier [114, 115]. The procyanidins in cocoa are unique because they exist as long polymers, prepared through polymeric condensation by two, three, or up to ten linked units of catechin or epicatechin [116] formed during fermentation [117]; thus, their favorable effects would be restricted to the gastrointestinal tract. These compounds represent 60% of the total polyphenol content in cocoa products [118, 119]. Cocoa and its products are generally consumed around the world because of highly attractive organoleptic characteristics [118]. Absolutely, cocoa and its derivatives constitute a larger proportion of the diet of many individuals than green tea, wine, or soybeans [118]. However, health benefits of cocoa flavonoids depend on their bioavailability (absorption, metabolism, and elimination) [120]. Of note, oligomers and polymers of flavanols that are not absorbed in the intestine can be metabolized by gut microbiota into various metabolites with low molecular weight, which tend to be well-absorbed through the colon and possess biological properties [121].

Intake of flavonoid-rich foods that possess antioxidant properties can have health effects [122]. Over the last few years, evidence emerged suggesting health benefits of cocoa phenolics, especially prominent for their metabolic and cardiovascular effects. These effects may be due to antioxidant and antiradical properties of cocoa bioactive compounds. Along with their antiplatelet effects [123], cocoa phenolics can be protective against heart diseases [124]. In addition, they have the capacity to modify the immune responses and produce anti-inflammatory and anticarcinogenic effects [125].

Below is an overview of evidences suggesting cocoa products as a cancer-protective factor. In particular, data from epidemiological studies support protective effects of cocoa and chocolate against cancer. Then, it would be also interesting to unravel potential biologic mechanisms through which cocoa phenolics can modify immune processes, thereby protecting against cancer. The focus is mainly to show anti-inflammatory and antioxidant effects of cocoa, which are known to decrease cancer risk. Inflammation provides a microenvironment appropriate for angiogenesis and therefore tumor growth [126]. Consistently, prospective studies have linked high levels of pro-inflammatory mediators such as IL-6, CRP, and TNF-α to increased risk of cancer in [127, 128]. An inflammatory response can result in the overproduction of ROS, which, in turn, would exacerbate the condition through oxidative stress.

Epidemiological Studies

Exposure to low doses of carcinogens may happen continuously during a lifetime. Furthermore, the body's response to carcinogens and chemoprotective agents depends upon several factors such as genetic polymorphisms and epigenetic modifications [129]. Few epidemiological studies have investigated the link between cancer-related mortality and cocoa, and consequently there is a limited support for the efficacy of cocoa for cancer-related mortality. Therefore, large-scale and long-term controlled trials are necessary to confirm cancer preventive effects of foodstuffs. Below provides a summary of existing studies by type. A review of epidemiological studies on polyphenols has previously addressed the link between catechin intake and cancer risk [130].

Case-Control Studies

Data supporting cancer preventive effects of cocoa in humans come mostly from the Kuna tribe in Panama. Kuna islanders drink flavanol-rich cocoa as their major cocktail. Studies have found lower mortality rates for cancer and other

chronic diseases among islanders than in mainland Panama. However, the finding should be treated with caution due to uncertainties arising from confounding factors [131]. Case-control studies have frequently investigated the relation between cocoa and cancer. They linked flavonoid consumption and procyanidin intake to decreased risk of gastric cancer [132]. In addition, higher catechin intake reported to be associated with lower rectal cancer incidence in postmenopausal women [133]; and higher consumption of epicatechin, anthocyanidin, and procyanidin was protective against non-Hodgkin lymphoma [134]. Although intake of these phenolic compounds has been associated with reduced risk of cancers [130, 135], the nutrition source for these bioactive compounds remains to be identified. Moreover, there are studies that failed to show the efficacy of cocoa intake in decreasing risk of cancer. For example, there was no relation between chocolate and cocoa intake and the incidence of any stage of colorectal diseases ranging from polyps and adenomas to CRC [136]. Lack of correlation might lie in the lower intake of flavanols (with a small percentage of cocoa-like milk chocolate) and/or low study power [136]. In another study, CRC risk was decreased by about 26% for epicatechin and by about 22% for procyanidins [136]. In a case-control study, procyanidins were associated with a lower risk of CRC. Interestingly, the higher the degree of polymerization of procyanidins, the lower the risk of CRC [137].

Cohort Studies

Four prospective cohort studies assessed the effect of cocoa and chocolate intake on mortality and cancer outcomes: Iowa Women's Study [133], the Zutphen Elderly Study from the Netherlands [138], the Harvard Alumni Study [139], and the Leisure World Cohort Study [140]. In the first study, no separate risk estimates of rectal cancer were shown for chocolate [133]. In the study [141], no association was found between chocolate intake and non-Hodgkin lymphoma, though total procyanidin consumption was protective, with a 30% lower hazard for the category with the highest consumption. Overall catechin consumption was associated neither with epithelial cancer nor with lung cancer after adjustment for confounders. However, nonsignificant inverse association was present between intake of catechins from cocoa and chocolate and incidence of lung and all epithelial cancers. In the Harvard Alumni Study, individuals who consumed candy 1–3 times per month had a 27% lower risk of mortality [139]. In the study [140], frequent chocolate consumption was not associated with lower mortality risk, but mortality seemed to decrease (about 6%) in people with occasional chocolate intake.

Intervention Studies

To our knowledge, no clinical trial on the effectiveness of cocoa and chocolate intake for cancer prevention is available. However, few human studies report that cocoa favorably affects intermediary factors in cancer progression, in particular inflammation and oxidative stress [142–145]. Recent studies focused on the modification of antioxidant and anti-inflammatory status by consumption of cocoa derivatives. One trial [146] has demonstrated that dark chocolate intake significantly improved DNA resistance against oxidative stress. Cocoa consumption reduced NF-κB activation in PBMCs of healthy volunteers [147]; but other biomarkers of inflammation, including IL-6, remained unaltered in a group of patients with cardiovascular diseases after cocoa powder intake [146].

Evidence for cancer chemoprevention by flavonoids comes from different study types. Antitumoral effects of flavonoids occur through induction of apoptosis and inhibition of several kinases and transcription factors, angiogenesis, and cell proliferation. Further, cocoa and its bioactive compounds have shown antitumoral effects independent of antioxidant function [115, 148, 149]. However, whether it works in humans remains to be addressed. Below different pathways and molecular targets whereby cocoa and their bioactive compounds interfere with cancer cells are reviewed.

Antioxidant and Antiradical Activities of Cocoa

Polyphenols are able to capture ROS which have been implicated in carcinogenesis. One serving of cocoa or chocolate has antioxidant capacity (AOC) that exceeds the antioxidant capabilities of many foodstuffs [118]. The cocoa procyanidins, epicatechin, and catechin have important antioxidant abilities [150, 151]. Genome analysis of human colon adenocarcinoma cell line (Caco-2 cells) revealed that polyphenolic cocoa extract can modulate the expression of numerous genes involved in cellular response to OS [152]. Phenolic compounds from cocoa inhibit lipid peroxidation in microsomes and liposomes. The polyphenolic cocoa extract increased mRNA levels, protein levels, and enzymatic activity of CYP1A1 in MCF-7 and SKBR3 breast cancer cells [153]. The cocoa polyphenolic extract led to inhibition of ROS generation and xanthine oxidase activity in stimulated myelocytic leukemia HL-60 cells [154]. In vivo studies also demonstrated the protective effect of cocoa in rodent models of CRC and lung cancer and liver injury [155, 156]. In a lymphoma model, the albumin fraction of semifermented dry cacao showed free radical scavenging capacity [157]. The cacao is, therefore, the source of potential antitumor agents. Upregulation of cytoprotective enzymes like Kelch-like ECH-associated protein 1 (Keap1) and its binding partner, transcription factor NF-E2-related factor-2 (Nrf2), which are involved in antioxidant response element (ARE), by therapeutic agents like cocoa and its phenolic compounds can subsequently activate ARE [158]. Epicatechin has been described to act through this pathway as well [159]. Human studies also showed similar results with an increase in plasma AOC and a decrease in plasma lipid oxidation [143, 160].

Cocoa and Immunity

Several studies of cocoa's effects on the immune system have been published in recent years. In vitro and in vivo models have investigated both the innate and adaptive immunity. Most in vitro experiments of cocoa and its components have focused on inflammatory mediators released by macrophages. Some studies tested the effects of cocoa administration in several models of inflammation. Human studies investigating the relation between cocoa and innate immune responses are scarce and provide inconsistent results. One study showed no significant effect of cocoa on inflammatory markers in a group of healthy subjects [142]. However, another study reported that regular intake of dark chocolate by healthy humans was inversely associated with serum C-reactive protein (CRP) concentrations [161]. In vitro, on cultured lymphoid cells or PBMCs, and in vivo models also have investigated the influence of cocoa on adaptive immune response.

An Overview of Inflammation in Cancer

Inflammation is a feature of innate immunity, and chronic inflammation is a contributing factor to the initiation and progression of cancer. Chronic inflammation acts as a trigger for premalignant and malignant transformation of cells. About 20% of all cancers are related to chronic inflammation resulting from infections and autoimmune diseases [162]. The association between inflammation and cancer involves key inflammatory mediators. Several inflammatory mediators, like NF-κB, TNF-α, and COX-2, have been related to cell proliferation, antiapoptotic activity, angiogenesis, and metastasis [163, 164]. Inflammatory cytokines and cells have been broadly recognized in cancers of the stomach, colon, skin, liver, breast, lung, and head/neck [165]. Inhibition of COX-2 and iNOS has shown protective effects against tumor development in animal models, suggesting that they are crucial targets for tumorigenesis. Inflammation can enhance mutation rates and proliferation of mutated cells. Inflammatory cells are sources of ROS that are able to induce genomic instability and DNA damage. More precisely, cells may use cytokines such as TNF-α to increase ROS in adjacent epithelial cells [166, 167]. On the other

hand, NF-κB, which regulates the expression of iNOS and COX-2, is constitutively active in neoplastic cells, posing a hazard to the development of cancer. The pro-tumorigenic function of TNF-α and IL-6 released by immune cells is well established. The role of TNF-α and IL-6 as master regulators of tumor-associated inflammation and tumorigenesis makes them striking targets for adjuvant therapy in cancer [163]. Diet can also contribute to chronic inflammation that facilitates the development of gastrointestinal cancers. Chronic consumption of alcohol activates mast cells, causes polyp formation, and enhances tumor formation and invasion in a mouse model of colon cancer. In addition, red meat contains high levels of N-glycolylneuraminic acid. This foreign antigen can get incorporated into tissue and attract inflammatory cells [165]. Inflammation can also modulate composition of the gut microbiota, assisting growth of harmful bacteria such as *Escherichia coli*, which are present in higher concentrations in patients with CRC. Colitis can cause tumorigenesis by changing microbiome toward a population more capable of inducing gene damage and mutagenesis [165]. Therefore, the use of chemopreventive substances that decrease inflammation seems to be a helpful approach to control the development and progression of cancers. For example, NSAIDs or selective blockers by inhibition of COX activity, which fuels cancer-related inflammation through prostaglandin E2, decrease the risk of some type of cancers including colon and lung cancer. However, further clinical studies are necessary to determine the possible benefits and risks of long-term NSAID use for cancer prevention and treatment [165]. For more information about the role of inflammation in cancer, see comprehensive reviews [162, 164, 168].

Anti-inflammatory Effects of Cocoa and Cancer

Different anti-inflammatory effects of cocoa extracts have been reported. Cocoa extract and EC decreased TNF-α, IL-1a, IL-6 expression and NO secretion in different cells. Cocoa phenolic extract inhibited phosphorylation of AKT and ERK induced by TNF-α and suppressed MEK1 (mitogen-activated protein kinase kinase-1) and phosphatidylinositol-3-kinase (PI3K) activity induced by TNF-α, suggesting a potential chemopreventive effect against pro-inflammatory cytokine-mediated skin cancer and inflammation [169]. Cocoa polyphenols reduced phosphorylation of TNF-α-induced c-Jun N-terminal kinase (JNK) and nuclear translocation of NK-κB [170]. High-molecular-weight polymeric procyanidins from cocoa decreased TNF-α-induced IL-8 in human colon cancer HT-29 cells [171]. Cocoa flavanols have demonstrated a critical role in the prevention of neoplastic lesions in CRC [172]. Feeding animals with a 12% cocoa-enriched diet suppressed intestinal inflammation induced by AOM through the inhibition of NF-κB signaling and downregulation of COX-2 and iNOS [170]. These effects suggest the chemopreventive effect of a cocoa-rich diet on colon inflammation and preneoplastic lesions. In another study, supplementation with dark chocolate decreased cell proliferation and downregulated transcription levels of COX-2 and RelA resulting in a lower number of preneoplastic lesions [173].

Other studies reported several possible immunological effects of cocoa and cocoa flavonoids on cancer models.

Cancer Immunity Cycle

The immune system is able to recognize tumor antigens. However, mechanisms for immune escape are a hallmark of cancer progression [174]. Antitumoral activity of the immune system involves different immune cells such as NK cells, dendritic cells (DCs), macrophages, and T-cells [175]. DCs capture tumor antigens, leading to activation and priming of effector T-cells (Teff) against tumor-specific antigens in lymph nodes [176]. DCs present antigens bound to major histocompatibility complex (MHC) molecules. Activated Teff infiltrate in tumor, recognize malignant cells, and kill them. DCs capture antigens from dying tumor cells, and this would trigger the cycle over again. Naïve T-cells cannot be

activated exclusively by recognition of cancer-specific peptide-MHC I complexes by T-cell receptor (TCR). Additional activator signals must be present involving pro-inflammatory cytokines (e.g., TNF-α, IL-1, IFN-α) [177], factors released by killed cancer cells such as high mobility group box 1 (HMGB1) and cyclic dinucleotide (CDN) [178], and Toll-like receptor (TLR) signaling. Because killing of cancer cells is accompanied by release of tumor-associated antigens and increased activation of Teff, it is expected that antitumor responses should occur repeatedly. However, different mechanisms help tumor cells to escape the immune system. For example, many tumors suppress MHC expression, thus masking their presence from TCR. In addition, after infiltration of Teff into cancer cells, activation of inhibitory signaling pathways in local microenvironment would reduce T-cell function. Inhibition of these pathways by immunological drugs removes cell intrinsic inhibitory pathways that block effective antitumor cell response [179–181].

Recent studies have suggested paradoxical roles of regulatory T (T_{REG}) cells in cancer [182]. FOXP3C CD4C CD25C/high T_{REG} cells are involved in the modulatory action of the immune system and, in particular, are valuable for coordinating control of peripheral immunological tolerance [183, 184]. The transcription factor FOXP3 is a critical regulator of T_{REG} cell function. T_{REG} cells provide the machinery for immune homeostasis during infections by inducing useful inflammatory responses while minimizing collateral tissue injury. However, T_{REG} cell function in cancers is widely regarded as negative [185–187]. In fact, an increased number of T_{REG} cells have been reported in patients with head and neck, pancreatic, stomach, breast, and liver cancers [181]. Tumor-associated T_{REG} cells pose a major challenge in vaccine therapy for cancers [185, 187, 188]. Therefore, several anti-T_{REG} regimens have been developed that rely on depletion of T_{REG} cells and inhibition of their suppressive function, their residence into tumors, and/or their differentiation/proliferation [185, 186]. For instance, anti-CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) immunotherapy that has shown promising results [189] depletes T_{REG} cells from tumor tissues [189]. Chronic inflammation mediated by cytokines and ROS may cause cell injury in target cells and therefore may contribute to cancer development. Mounting evidence suggests that tumor-associated inflammation is a tumor-promoting event. The reason is that inflammation can support cancer cell survival through DNA damage and development of a tumor stroma.

Almost immediately after birth, the gastrointestinal (GI) tract changes from sterility to a large ecosystem with hundred trillion microbial organisms, representing the most densely populated ecosystem known so far [190]. The overall population of intestinal colonies including bacterial, fungal, and viral communities is referred to as the gut microbiota. The microbiota include more than 1500 bacterial species, which are estimated to encode more than 150 times more genes than human genome. The gut microbiota is in intricate and reciprocal interaction with the human host and nutrients, providing a metabolic engine important for GI health and disease. This highly regulated and complex ecosystem plays an important role in priming the immune system and maintenance of intestinal immune homeostasis [191, 192]. Besides metabolic effects, the gut microbiota affects tissue development and inflammation [193–196]. Providing a physical barrier against pathogens and supplying immunological surveillance signals are other functions of the gut microbiota. There should be an ability to maintain the balance between tolerance toward microbiota and surveillance against pathogens. Such ability comes from the cross talk between the intestinal microbiota and host that involves both innate and adaptive immunity [197–199]. The hygiene hypothesis reflects the fact that the lack of exposure of the gut to harmless microorganisms, called “old friends,” in infancy causes certain deficiencies in the immune system at later age. A number of immunological disorders such as allergic diseases, inflammatory bowel diseases, type 1 diabetes, and multiple sclerosis are thought to result from an imbalance in the function of the regulatory immune system. Proper discrimination between harmful and

harmless pathogens involves a family of cell surface and cytosolic receptors of the innate immunity, called pattern recognition receptors (PRRs). PRRs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and nucleotide-binding oligomerization domain proteins (NOD proteins) recognize PAMPs and damage-associated molecular pattern (DAMPs). Interestingly, both harmful and harmless bacteria express these PAMPs. In this manner, pathogenic bacteria can pass through the epithelial barrier and activate inflammatory cascade through increased NF-κB translocation, on one side, and on the other side, commensal bacteria do not invade epithelial cells and do not stimulate inflammatory responses [200–203].

Commensal bacteria colonization results in Paneth cell expression of an antimicrobial peptide, regenerating islet-derived 3 gamma (Reg III-γ) [204, 205], which is involved in innate immune protection against enteric pathogens [206]. Moreover, the gut microbiota helps with maintaining the balance between Teff and Treg cell function. Expansion and differentiation of T-cells into T_{REG} cells occur in the colon in the presence of microbiota. T_{REG} cells suppress inflammatory response via the production of anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β. A mixture of *Clostridia* strains induces the accumulation of T_{REG} cells in the colon and expression of IL-10 and CTLA-4 by Treg cells. *Lactobacillus reuteri*, *L. murinus, and Helicobacter hepaticus* have also been implicated in T_{REG} function [207–210].

Cancer Prevention and Treatment, Immunity, and Probiotics

Epidemiological Studies

The preventive effect of probiotics or fermented products containing probiotics on animal and human cancers has been frequently investigated. However, epidemiological evidence is scarce. Studies on humans showed a reverse association between yogurt intake and the risk of breast cancer [211]. In another case-control study in the United States, yogurt consumption was reported to be protective against colon cancer [212]. Similar results were found by Dutch researchers for breast cancer [213]. There are clinical trials investigating the possible role of probiotics in cancer prevention. In one trial, recurrence rate of superficial bladder cancer was lower in subjects who received *L. casei* Shirota (LcS) in comparison with subjects receiving placebo [214]. However, it must be clarified if long-term supplementation of probiotics can significantly reduce the risk of CRC in humans. A cohort study with 12 years of follow-up on 45,241 volunteers determined that high yogurt consumption was significantly related to lower risk of CRC [215]. However, these studies have limitations concerning selection and standardization of microorganisms, control of food intake, time, and dosing of microorganism administration.

Clinical studies have also investigated the beneficial effect of probiotics in preventing GI disorders, including viral diarrhea and chemotherapy, radiotherapy, or antibiotic-associated diarrhea. In addition, chronic treatment with probiotics effectively reduced the urinary excretion of aflatoxin B(1)-N(7)-guanine (AFB-N(7)-guanine), a marker for hepatocyte carcinogenesis, and the risk of CRC [216]. It is commonly believed that probiotic supplementation can decrease the risk of breast cancer in perimenopausal women. However, clinical studies report inconsistent results. It seems that long-term use of probiotics is needed to achieve chemopreventive effect on the development of malignant tumors. For instance, *L. casei* supplementation for 4 years could prevent atypical CRC [217]. Usual consumption of *L. casei* Shirota (LcS) and soy isoflavone in adolescents was inversely related to the incidence of breast cancer in Japanese women [218]. In contrast, the 3-month yogurt consumption could not improve cell-mediated immune function in women [219]. Altogether, evidence for the efficacy of probiotics in human tumorigenesis is ambiguous. However, there is mounting evidence from experimental models indicating antineoplastic effects of probiotics. In addition, as shown through a meta-analysis study, data from epidemiological

studies reveal a decreased risk of CRC and precursor lesions in association with consumption of probiotics. However, interventional studies are necessary to confirm the efficacy of probiotics [220]. Coupled to the above is the need for long-term high-quality studies that assess the efficacy of probiotics in subjects with different types and stages of cancer.

Cancer Prevention

Study of proto-oncogene human epidermal growth factor receptor-2 (HER2)/neu-driven transgenic mice showed that extended contact to metronidazole in combination with ciprofloxacin increases the risk of breast cancer [221]. In fact, altered composition of the gut microbiota may influence the development and progression of cancer through inflammatory and metabolic pathways [222, 223]. However, not all probiotics have the ability to modulate the immune system and thereby play a role in cancer prevention. Previously, it has been reported that a dose of 10^8–10^9 colony-forming unit (CFU)/day of a strain with immunomodulatory effect and a duration of 48–72 h is required to influence the host immune homeostasis [224, 225].

Maintenance and Enhancement of Intestinal Barrier Function

Mucin 2 (MUC2) is the main mucin secreted by intestinal goblet cells. MUC2-deficient mice have increased risk for CRC [226]. Treatment with probiotics was reported to be effective to promote the restoration of colonic tissue through an increased MUC2 expression, extracellular mucin secretion, and inhibition of enteropathogenic adherence. Increased epithelial permeability has been implicated in early stages of CRC. *L. plantarum* MB452 was shown to enhance Caco-2 tight junction (TJ) integrity, possibly through encoding TJ-related genes including occludin and scaffold protein zonula occludens [226–228]. Probiotics are also capable of preventing epithelial barrier damage by stimulating the production of cytoprotective heat shock proteins in stressed epithelial cells to maintain hemostasis [229] and promote cell survival [230]. Components of *E. coli* strain Nissle 1917 can decrease intestinal permeability by restoring a disrupted epithelial barrier [231]. Combination of *L. rhamnosus* GG (LGG) and *B. lactis* Bb12 could also improve epithelial integrity in patients with CRC [232].

Recognition of Probiotics by the Immune System: Toll-Like Receptors

TLR2 plays a protective role in colitis-associated CRC. TLR2-deficient mice demonstrated increased inflammation and elevated serum levels of inflammatory markers such as IL-6 and IL-17A. Probiotics can modify the risk of CRC through a TLR2-dependent pathway. TLR2 recognizes gram-positive bacteria, such as lactobacilli and bifidobacteria [233, 234]. Peptidoglycan from lactobacilli blocks the production of pro-inflammatory cytokine IL-12 by macrophages via TLR2 [235]. In addition, mixture of *L. plantarum* and *L. casei* synergistically stimulate IL-10 production in macrophages through a TLR2-dependent pathway [233].

Modulation of DCs

IL-10 suppresses the production of pro-inflammatory cytokines while promoting the development of T_{REG} cells. Studies showed a strong association between probiotics and induction of IL-10 by DCs [236]. Administration of probiotics also induced regulatory DCs, which, in turn, could promote the induction of CD4+Foxp3+ T_{REG} cells in vivo [237]. Thereby, mice showed a reduction in the production of pro-inflammatory cytokines IL-17, IFN-γ, and TNF-α and an amelioration of disease progression. In the study [238], the authors investigated the ability of three *Lactobacillus* species to influence DC to drive T_{REG} cell development. Human monocyte-derived DC matured in the presence of *L. rhamnosus* showed decreased capacity to support T-cell proliferation and attenuate CD3/CD28-induced cytokine production.

L. rhamnosus GG, *B. lactis* Bb12, and/or inulin enriched with oligofructose demonstrated immune stimulatory effects by inducing the maturation of DC [239], supporting the immune response against tumor cells [240]. Activation of IL-10-secreting cells was accompanied by the induction of apoptosis in colon cancer cells and suppression of pro-carcinogenic factors [241]. *Bifidobacterium* in a mice model has been shown to alter DC activity, leading to improved tumor-specific $CD8^+$ T-cell function.

NK Cell Proliferation and Activity

Probiotics are also able to increase NK cell numbers and their cytotoxic activity [242]. Oral administration of *L. casei* Shirota (LcS) to tumor-bearing mice stimulated splenic NK cell activity, thus leading to postponed tumor formation [243–245]. Probiotics exert desmutagenic effects on myeloid DC maturation through IL-12 production and shifting T-cell activity toward T_H1, T_H2, or even T_{REG} type of responses [244, 246, 247]. Later, this molecule, IL-12, activates NK cells to produce IFN-γ [248]. In this manner, LcS is able to suppress murine tumorigenesis via increased IL-12 production by bone marrow-derived cells in vitro [249] and inhibition of IL-6 production in the colonic mucosa [250, 251]. Previous studies demonstrated that *Lactobacillus* strains with a firm cell wall resistant to intracellular digestion can stimulate high levels of IL-12 [252].

Lactobacillus and *Bifidobacterium* strains and their mixture differentially initiated NK/DC interactions via induction of DC maturation and cytolytic potential of NK cells [253]. NK cells play a critical role in tumor surveillance and production of IFN-γ and TNF-α, which induce cell-mediated immunity and lead to further activation of APCs (DCs and macrophages) [248]. NK cells also are indirectly activated by DCs which secrete soluble factors, such as IL-12, IL-18, and type I interferons. Probiotic *Lactobacillus* strains can induce secretion of pro-inflammatory cytokines, IL-12 and TNFα [254], which are positively correlated with NK cell activity.

IL-12 produced by DC and APC primes NK cell activation and subsequent secretion of TNF-α. Therefore, LcS and *Lactobacillus* strains may indirectly activate NK effector cells through DCs and APCs, respectively. NK-derived IFN-γ secretion has been implicated not only in innate antitumor immune responses but also in cell-mediated antitumor immune responses [253, 255]. In one trial, intake of fermented milk containing LcS enhanced NF-κB activity in subjects. The effect was reduced in the presence of anti-IL-12 monoclonal antibody [256]. DCs, T_{REG} cells, and NK cells are important immune cells in defense against cancer [251, 257]. However, supplementation with synbiotics containing LGG, *B. lactis*, and oligofructose for 12 weeks showed little effects on systemic immune responses in patients with CRC [239].

Inhibitory Effect of Probiotics on TLR4 and COX-2 Expression

COX-2 has been implicated in inflammatory diseases and CRC. TLR4 is mandatory for the induction of COX-2 and therefore CRC development [258]. Overexpression of TLR4 upregulates NF-κB activation and COX-2 expression [259]. The probiotic combination VSL#3 has been reported to downregulate COX-2 expression in Colo320 and SW480 intestinal epithelial cells (IECs). COX-2 has been associated with an increased risk of CRC because it stimulates cell proliferation and triggers inflammatory pathways [35]. Milks fermented with different strains of probiotics have been investigated in HT-29 colon cancer cells. Almost all of them induced a significant, although variable, reduction in the growth of HT-29 cells [260, 261].

Probiotics Enhance Innate Immune Functions

Defensins through membrane lysis and DNA damage exert cytotoxic activity on tumor cells. Murine b-defensin 2 has been shown to promote DC maturation, which initiates type I polarized immune responses through the production of pro-inflammatory cytokines such as IL-12, IL-1α, IL-1β, and IL-6 [262]. Treatment of Caco-2 colorectal adenocarcinoma cells with *L. plantarum* through the induction of TLR2 significantly upregulated the mRNA expression and secretion of human b-defensin 2 (HBD-2) in a

dose-dependent manner [263]. A probiotic mixture, including several *E. coli* strains, VSL#3, and lactobacilli, increased HBD-2 synthesis in human and Caco-2 cells [264]. In addition, probiotic products enhanced host immune function by increasing phagocytic activity of macrophages [265].

Immunoglobulins

IgA exerts anti-inflammatory and also cytotoxic effects on tumor cells [266]. It is resistant to proteolysis and can limit contact between potentially carcinogenic compounds and colon cells [225]. A study of mice treated with carcinogen showed that consumption of yogurt containing probiotics was efficient to downregulate cancer progression in the large intestine through upregulation of IgA, T-cell function, and colonic macrophage activities [234]. However, the effect of probiotic supplementation on the production of IgA remains controversial [265, 267]. LcS has been shown to inhibit tumor development and IgE production in mice [268].

Administration of *L. acidophilus* SNUL, *L. casei* YIT9029, and *B. longum* HY8001 improved the survival of tumor-bearing mice. The effect was associated with enhanced cellular immune responses as reflected in increased numbers of total T-cells, NK cells and MHC class II^+ cells, and $CD4^-CD8^+$ T-cells [269]. *Lactobacillus rhamnosus* strain GG (LGG) was reported to delay the onset of cancer through mitigating CD3 T-cell depletion in tumor-bearing mice while enhancing activation of CD8 and $CD4^-$ T-cells without significant effect on NK cell function [270]. Furthermore, *L. acidophilus* suppressed MHC class I expression and also induced a decrease in mRNA expression of stromal-derived factor-1 receptor, CXCR4, suggesting a role in cancer metastasis prevention [271]. In DSS-induced CRC mice, *Lactobacillus* and the VSL#3 mixture increased levels of angiostatin, an endogenous inhibitor of angiogenesis and regulatory T-cells [272]. In contrast, there was an increase in the number of memory $CD4^+$ T-cells and pro-inflammatory cytokines IL-17 and TNF-α [272].

Pre-inoculation with *L. plantarum* significantly reduced tumor growth and activated innate immunity while increasing the intratumoral levels of $CD8^+$ T-cells and NK cells in the tumor microenvironment [273]. Probiotic administration significantly increased the $CD8^+/CD4^+$ T-lymphocyte ratio. CD4 cells induce production of cytokines such as IL-6 and IL-10. Thus, increasing the CD8+/CD4+ T-lymphocyte ratio might explain lowering of IL-6 and delayed tumor growth by probiotics [274]. Indeed, *L. reuteri* was shown to delay the onset of neoplastic features through the induction of anti-inflammatory $CD4^+CD25^+$ T_{REG} cells. Stimulated T_{REG} cells would direct immune networks in a manner to resist against inflammatory diseases, including early stage of malignant transformation [275]. *L. rhamnosus* GG has been demonstrated to be effective in reducing the recurrence of bladder cancer [276]. The effect may be mediated by increased levels of chemokine (C motif) ligand (XCL1); this chemokine produced by activated $CD8^+$ cells and γδ-T-cells, NK cells, and master cells, which helps in chemotaxis by T-cells and NK cells and thus assists in tumor regression [276]. Activation of phagocytes by probiotics can inhibit cancer cells in early stage. Kefir consumption caused stimulation of phagocytes present in Peyer's patches and in the peritoneum [277].

Researchers have argued that stimulation rather than suppression of the innate immune system can contribute to cancer development. Yogurt feeding was correlated with altered levels of cytokines, such as TNF-α, IFN-γ, and interleukins [278, 279]. Intrapleural injection of LcS in mice could improve immunity against tumor development through release of TNF-α, an antitumor agent. In line with these observations, other studies also noted that intrapleural administration of LcS in tumor-bearing mice induced the production of IFN-γ, IL-1β, and TNF-α, leading to the inhibition of tumor growth and therefore an increased survival [280, 281]. Similar results have been reported for *L. acidophilus* SNUL, *L. casei* YIT9029, and *B. longum* HY8001 strains [269]. Urbanska and colleagues [279] investigated the effect of microencapsulated probiotic *Lactobacillus acidophilus* in a model of CRC. Daily oral administration of the microorganism significantly

induced suppression in tumor growth, tumor multiplicity, and tumor size. In a study by de Leblanc et al. [282], LcS induced the secretion of inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ, resulting in reduced tumor development and improved survival of mice treated with a carcinogen [281]. IFN-γ is involved in activation of NK cells and macrophages. Consequently, it plays a significant role in cancer prevention. Humans and animals continuously produce IFN-γ in the defense against cancer [283]. Excessive inflammatory response is not desirable, and probiotics are able to induce and control T_{REG} cell function [284]. Direct immune modulatory effects of *B. lactis* and *L. rhamnosus* have been reported to be mediated through reduction of IL-2 and inducible NO synthase [285, 286]. Antitumoral and immunoregulatory effects of LcS have been investigated in various models. Of note, oral administration of LcS has demonstrated antitumoral activity against bladder cancer cells in clinical trials [287].

Modulation of Inflammatory Response

Chronic inflammation has been recognized as a risk factor for cancer. Inflammation plays a causative role in colitis-associated colon cancer, sporadic colon cancer, and hepatocellular carcinoma (HCC) [288–290]. Previous studies have reported antitumoral and anti-inflammatory effects of probiotics [291, 292]. LGG was reported to prevent colon cancer, accompanied by suppression of NF-κB pathway [293]. Li et al. showed a reduction in the level of IL-17 by probiotics in an HCC model. It suggests an association between immunomodulatory and antitumoral effects of probiotics [290]. Mounting evidence suggests the IL-6-lowering effect of *L. casei* CRL431. The proangiogenic role of IL-6 is consistent with impaired tumor growth by probiotic supplementation [274].

The *Lactobacillus casei* BL23, recognized for its anti-inflammatory characteristics, was tested for its protective effects on CRC in mice [294]. Mice in probiotic group substantially showed reduced levels of the monocyte chemoattractant protein-1 (MCP-1) and TNF-α with high levels of anti-inflammatory ones, such as IL-10 [294].

IL-17A produced by T_H17 cells would assist angiogenesis. Although the role of T_H17 cells and IL-17 in cancer is still inconsistent, but it has been suggested that reduction of T_H17 cell population and IL-17 level may inhibit progression of cancer [295, 296]. Noteworthy, ex vivo studies on splenic cells incubated with *L. casei* BL23 showed reduced numbers of T_{REG} cells and increased percentage of T_H17 cells and higher production of IL-17, IL-6, and TGF-β, together providing a microenvironment favorable to T_H17 differentiation [294]. As mentioned before, a probiotic mixture led to reduction in the proportion of T_H17 cells and in the production of IL-17 in an HCC model. In contrast, *L. casei* BL23 caused an increase in the proportion of T_H17 cells and in the production of IL-17 in a model of CRC [290]. However, both studies revealed an increase in the levels of anti-inflammatory cytokine IL-10 and anti-angiogenic cytokine IL-22. This would reflect a T_H17-mediated response.

IFN-γ plays a role in cancer immunity by increasing MHC I expression, T-cell infiltration, differentiation to cytotoxic T-lymphocytes, and T_H1 polarization, orchestrating different antitumoral immune responses [297, 298]. IFN-γ also has been used clinically for its antitumoral effect, leading to improved survival. Studies of mice reveal the role of IFN-γ in mediating the protective effect of probiotics against cancer [299, 300].

Production of Active Compounds Which May Be Involved in Immunity

Short-chain fatty acids (SCFAs) are the products of bacterial fermentation of nondigestible carbohydrates. Butyrate is a SCFA that can contribute to cancer prevention in different ways. It has the ability to increase mucus production and improve intestinal barrier function. It is also able to stimulate the production of anti-inflammatory cytokines, such as IL-10, while decreasing the production of pro-inflammatory cytokines by inhibiting the activation of NF-κB. More interestingly, butyrate can increase the immunogenicity of tumor cells by monitoring neutrophils and antigen-presenting cells and through regulation of chemotaxis by neutrophils, DCs, and macrophages [301] and suppressing COX-2 activity

[302, 303]. Other SCFAs like acetic and propionic acids also exhibit the same anti-inflammatory activity through suppression of NF-κB signaling pathway [304, 305].

Some species of probiotic bacteria, such as *Lactobacillus acidophilus*, are able to produce conjugated linoleic acid (CLA) from linoleic acid. CLA can suppress the production of eicosanoids in colon cells through replacement of arachidonic acid by CLA in the cell membrane and through interference with cyclooxygenase and lipoxygenase (LOX) enzymes. Probiotic supplementation can increase the production of CLA to promote antitumor immunity in a dose-dependent manner [241, 306].

Immunological Effects of Probiotics Combined with Chemotherapy

Probiotics also can be used in combination with conventional cancer therapies. In particular, disruption of the gut microbiota can impair the cancer cell response to platinum salts as chemotherapy. Supporting this, mice treated with an antibiotic mixture (including vancomycin, imipenem, and neomycin) displayed reduced therapeutic response to oxaliplatin and cisplatin in a colon carcinoma (MC38) and lymphoma (EL4) model, respectively. Interestingly, it has been reported that combination antibiotic therapy reduces oxaliplatin-induced DNA damage and apoptosis in tumor-bearing mice. In addition, *Ruminococcus*, *Alistipes*, and *Lactobacillus fermentum* are capable of affecting tumor response to CpG oligodeoxynucleotide (ODN), probably through regulation of TNF production [307, 308].

The study [309] proved that the efficacy of cyclophosphamide as an anticancer immunomodulatory agent, at least in part, relies on the gut microbiota. Tumor-bearing mice that were either germ-free or antibiotics-treated showed a reduction in "pathogenic" T-helper (pT_H17) responses, and their tumors were more resistant to cyclophosphamide-based therapy. It seems that this cyclophosphamide would stimulate pT_H17 cells through a complex circuitry that involves the gut microbiota [309]. More precisely, treatment with cyclophosphamide causes a reduction in the abundance of lactobacilli and enterococci in the gut [309]. Gram-positive bacteria, such as *L. johnsonii* and *E. hirae*, promote differentiation of $CD4^+$ T-cells into T_H1 and T_H17 cells. Broad-spectrum antibiotics suppressed cyclophosphamide-induced production of IL-17 and IFN-γ [309]. Consistently, another study [310] showed that two bacterial species, *Enterococcus hirae* and *Barnesiella intestinihominis*, are involved in response to cyclophosphamide therapy. After cyclophosphamide treatment, *E. hirae* migrates to secondary lymphoid organs, followed by mounting pT_H17 immune responses and accumulation of IFN-γ^+ IL-17^+ cells and $CCR6^+$ $CXCR3^+$ $CD4^+$ T-cells and T_{REG} cells in the spleen [309].

Studies have demonstrated the significance of *Bifidobacterium* to natural antitumor immunity and also in response to anti-PD-L1 antibody therapy and CTLA-4 therapy in tumor settings [311, 312]. Furthermore, *Bacteroides fragilis* improved response to CTLA-4 blockade, by affecting IL-12-dependent T_H1 immune response. *Bifidobacterium* in combination with anti-PD-L1 antibody enhanced antitumor immunity through activation of DCs [312].

Altogether, finding bacterial genera linking intestinal immune homeostasis and anticancer immune responses is essential to shed light on the possibility of using selected bacteria to improve cancer therapy by enriching the gut microbiota. In patients with metastatic melanoma, an increased delivery of bacteria belonging to the *Bacteroidetes* phylum is associated with an increased resistance to the development of checkpoint blockade-induced colitis [313]. Recent advances in this field such as fecal transplant open up new avenues in cancer therapy [314, 315].

Role of Microorganisms in the Development of Cancer

Tumorigenesis is a complex process. As a result, it is difficult to draw a direct association between dysbiosis, inflammation, and tumorigenesis.

Adherent/invasive *E. coli* strains are present in great quantity on the colonic mucosa of patients with CRC but not normal colonic mucosa. This indicates involvement of *E. coli* colonization in cancer pathophysiology [316]. Long-term colonization of enterotoxigenic *Bacteroides fragilis* (ETBF) led to colitis and multiple intestinal neoplasia (MIN) in mice [317]. On the other side, IL-10-deficient mice colonized with *Bacteroides vulgates* displayed low-grade inflammation and more interestingly were less likely to develop colorectal tumors as compared with conventionalized IL-10-deficient mice [318]. The results support the differential role of gut microorganisms in intestinal immune homeostasis and CRC. There is a complex interaction between the gut microbiota and IECs, where innate immune receptors including Nod-like receptors (NLRs) and TLRs play a role. It has been reported that Nod1 pathway could increase tumor-promoting effect of attenuated Wnt signaling. Furthermore, gut microbiota depletion by antibiotics decreases tumor development in Nod1-deficient mice [319]. These data highlight the complicated interaction between the microbiota, inflammation, and cancer and support the hypothesis that susceptibility to cancer would be influenced by the composition of the gut microbiota and by the repertoire of host innate sensors as well. As a result, modification of the intestinal microbiota using probiotics or prebiotics may affect the development of cancer.

Gut Microbiota Induces Potent T_{REG} Cells with Systemic Antineoplastic Properties

The association of tumor-associated cells expressing T_{REG} cell markers including FOXP3 with poor prognosis of human cancers remains inconsistent. Under certain conditions, microbial priming of T_{REG} cells not only protects against cancer development but also helps remission of already established intestinal, mammary, and prostate cancers [320]. However, T_{REG} cells play paradoxical roles in cancer [320, 321]. Actually, Treg-mediated decreased risk of cancer is dependent on microbiota-induced IL-10, which acts to maintain immune system homeostasis and support a protective anti-inflammatory and antineoplastic T_{REG} phenotype. Probiotic consumption in mice shifts immunity toward IFN-γ and CD25 to improve wound healing and promote systemic health [322]. IFN-γ levels increase during T_{REG}-mediated tumor regression in mice. Recent findings show that an unbalanced gut flora would weaken response to immune [307, 309] and non-immune chemotherapeutic regimens such as cisplatin and oxaliplatin [307].

Based on the "hygiene hypothesis," hygienic subjects are vulnerable to a redirection of unbalanced resting peripheral T_{REG} to T_H17 immune responses, putting them at higher risk of autoimmune diseases and cancer [182]. Furthermore, consumption of beneficial probiotic bacteria led to the expansion of $Foxp3^+$ cells in the periphery [275, 322], improving defense against mammary cancer [275]. Probiotics-induced enhancement of the T_{REG}-dominated arm of the immune system did not interfere with the capability to respond against invading pathogens [322]. Altogether, the gut and its cross talk with the host determine the fate of preneoplastic and neoplastic lesions arising in epithelia throughout the body. It would open up a new avenue in cancer immunotherapy through modulation of beneficial T_{REG} via diet. This concept not only could be considered for fighting cancer, but also arousing these dormant T_{REG}-mediated capabilities may give an alternative approach to reduce cancer risk and promote overall good health and longevity [320].

Lactoferrin, Immunity, and Cancer

Lactoferrin (Lf) is an iron-binding glycoprotein belonging to the transferring family. It contributes to the regulation of iron absorption in the bowel and immune responses, as well as is able to exert antimicrobial, antioxidant, antitumoral, and anti-inflammatory effects [323, 324]. Lf is produced by mucosal epithelial cells and is present in most biological fluids, including tears, saliva, vaginal fluids, semen, and most abundantly milk and colostrum [324]. Moreover, it is

present in considerable amounts in polymorphonuclear granules [323]. Recent reports have shown that this multifunctional agent essentially exerts antimicrobial effect, which can be directed against bacteria, fungi, and viruses [325]. Other Lf-mediated activities include immune modulatory functions and tumor growth inhibition [325]. Its bacteriostatic effect is mediated through iron-binding ability, which consequently restricts the use of iron by bacteria and inhibits their growth systemically. Additionally, Lf damages the external membrane of the gram-negative bacteria by interacting with the lipopolysaccharide (LPS) [323]. Therefore, knowledge of the physiological role and possible therapeutic implications of LF is hastily growing. Here, we present possible antitumoral effects of LF through immune modulatory activity.

Antitumor Activity

The first reports suggesting that Lf may possess antitumor effects through depleting tumor cells of glutathione, making them more susceptible to chemotherapy, appeared in 1995 [326]. Since then, in vitro studies have demonstrated antitumor effects of Lf in different cancer cell lines such as breast cancer [327, 328], pancreatic cancer, colon cancer, and oral squamous cell carcinoma [329–331]. Suggested mechanisms include increased NK cell cytotoxicity and inhibition of cell growth and metastatic colony formation. Chemopreventive effects of bovine Lf (bLf) also have been implicated in treatment of tumors of the colon, peritoneum, lung, esophagus, mouth, and neck. Moreover, the immune modulatory effect of Lf has been shown in mice [332–334]. Oral administration of recombinant human Lf has been investigated in head and neck squamous cell carcinoma in mice. Animals treated with Lf exhibited tumor growth inhibition of 75% concurrent with a 20-fold increase in lymphocyte ratio compared with controls. Of note, when mice were depleted of CD3+ cells, Lf-induced tumor inhibition was abrogated [335].

Other studies investigated the effects of iron-saturated (i-s) bLf on the augmentation of chemotherapy. Results showed that chemotherapy eradicated large lymphomas only in mice fed 100% i-s bLf for at least 2 weeks prior to chemotherapy, but not in mice fed lower saturated forms of bovine Lf or control mice fed no bLf. Lf was nevertheless effective in augmenting chemotherapy at the lowest dose tested, equated to a 70 kg person ingesting 3 grams of Lf per day. In addition, 100% i-s bLf decreased angiogenesis, increased apoptosis, and supported immunomodulation, as reflected in increased production of T_H1 (TNF-α, IFN-γ, and IL-18) and T_H2 (IL-4, IL-5, IL-6, and IL-10) cytokines, which are necessary for optimal antitumor immune responses. Moreover, 100% i-s bLf also restored both RBC and WBC numbers depleted by chemotherapy [336]. However, the ability of Lf to exert a protective effect at sites far away from the GI tract is less understood [337].

Evidence for Chemopreventive Potential

Anti-inflammatory Activity

Lf possesses potent modulatory properties. It can decrease the production of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor (GMCSF)) [335, 338–341] while upregulating the levels of anti-inflammatory cytokines (IL-10) [342, 343]. However, other studies reported inconsistent results: (1) ex vivo upregulation of TNF-α and IFN-γ concurrent with a reduction in IL-5 and IL-10 upon induction with the exotoxin toxic shock syndrome toxin-1 [344] and (2) enhanced IL-12 production and decreased IL-10 release in human immunodeficiency virus (HIV)-infected children [345]. Together, these results indicate that Lf affects the T_H1/T_H2 cytokine balance independent of the host immune setting. Thus, Lf can increase the production of T_H1 cytokines in settings requiring an augmented ability to control infection on one hand and on the other hand may decrease the production of T_H1 cytokines to restrict excessive inflammatory responses [346]. Moreover, intravenous administration of bLf 24 h presurgery eased thymec-

tomy- and splenectomy-induced TNF-α and IL-6 generation, suggesting that Lf may have therapeutic application in cases of shock syndromes [347].

Immune Modulatory Activity

As already discussed, Lf also possesses immune-modulating properties. In vivo studies on the oral administration of bLf in mice revealed increased levels of NK cells, $CD4^+$ and $CD8^+$ cells, and IFN-γ^+ cells, in both the mucosal layer of the small intestine and the peripheral cells [348–350]. In addition, NK cell cytotoxicity is increased both in vitro and in vivo [328, 351, 352]. In humans, $CD3^+$, $CD4^+$, and $CD8^+$ T-cell activation has been observed as well [353].

Endogenous Lf belongs to the innate nonspecific immune system. However, mounting evidence shows that it may also be attributed to acquired immunity and protection against inflammation. As a powerful modulator of inflammatory and immune responses, Lf supports protection against both microbial infections such as septic shock and inflammatory diseases such as arthritis, chronic hepatitis, and cancer [354–356]. The modifying activity by Lf is connected to its capability to interfere with both specific cell receptors on a wide range of epithelial and immune cells [357] and pro-inflammatory bacterial components like LPS [358].

At the molecular level, the modulatory characteristics of Lf are mediated through iron binding and interactions with a multitude of compounds such as LPS. At the cellular level, Lf modifies the migration, maturation, differentiation, activation, proliferation, and function of immune cells. Some possible mechanisms include modulation of NF-κB and MAP kinase signaling [354]. Lf has been shown to increase the accumulation of neutrophils to sites of damage, support cell-to-cell interaction by promoting "stickiness," promote phagocytosis by polymorphonuclear leukocytes (PMNs) and monocyte/macrophages, support motility and superoxide production, reduce the release of pro-inflammatory cytokines, increase the number and activity of NK cells, and promote the maturation of lymphocytes [359–363].

In addition, a mechanism underlying antitumor effect of Lf is regulation of NK cell activity [328, 364] and inhibition of vascular endothelial growth factor (VEGF)-mediated angiogenesis [365]. It was reported that Lf has a significant effect on NK cell cytotoxicity and target cell sensitivity to lysis in hematopoietic and breast epithelial cell [328]. Other studies reported inhibition of tumor growth and lung colonization by B16-F10 melanoma experimental metastasis in mice treated with human Lf through increased NK cell activity [351].

Rodent cancer models have shown enhancement of intestinal immune homeostasis following oral administration of Lf. In particular, increased activation of NK cells, $CD4^+$ T-cells, and $CD8^+$ T-cells was demonstrated after Lf administration [348, 349].

In vivo oral administration of bLf enhanced NK cell activity and $CD4^+$ and $CD8^+$ T-cells in tumor-bearing mice [349, 350, 362] and also increased $CD3^+$ and $CD4^+$ T-cells in immunocompromised mice [366]. Activation of $CD4^+$ T-cells induces the generation of plasma B-cells, memory B-cells, and antibodies [367, 368]. Moreover, $CD4^+$ activation improves macrophage function, by inducing the release of cytokines [346]. Further activation of $CD4^+$ T-cells induces the generation of cytotoxic $CD8^+$ T-cells, which would destroy virus-invaded cells, cancer cells, and intracellular bacteria, as indicated in experimentally induced cancers [369].

Inhibition of Angiogenesis

Administration of bLf was reported to inhibit angiogenesis in rats [365] and mice [370]. In contrast, human Lf promotes angiogenesis [371]. BLf may inhibit angiogenesis through inhibition of IL-18 production [372]. Moreover, increased levels of IL-18 raise mucosal and systemic immune responses via cytokine secretion and NK cell activation [373]. In addition, Lf can reduce the levels of pro-inflammatory cytokines such as IL-6 and IL-1β as potent angiogenic inducers [374].

Clinical Trials

Few studies investigated the effect of lactoferrin on the immune system. In one study, 2 g bLf/day for 4 weeks increased phagocytic activity of

PMNs in three participants and increased $CD16^+$ T-cell counts in two of them. There was an augmentation in the percentage of NK cells, the percentage of $CD11b^+$ and $CD56^+$ T-cells, and the $CD16^+$ cell counts [375]. The oral administration of 40 mg bLf equivalent/day for 10 days in healthy participants resulted in an increased percentage of lymphocytes and immature cell forms, concurrent with a reduced percentage of neutrophils, eosinophils, and monocytes. Additionally, TNF-α levels were reduced, while changes in IL-6 were not significant [376]. The oral administration of placebo, 2, 10, or 50 mg of Lf daily, for 7 days in healthy subjects exhibited a significant, though transient, increase in the number of immature neutrophils and a significant reduction in the release of IL-6 and TNF-α by peripheral blood cells [377]. It has been suggested that a function of Lf could be to modify inflammatory reactions through the regulation of cytokine generation [378, 379].

Antitumor Immunity and Dietary Components

About ten plant-derived anticancer drugs are currently approved. They can be classified into four main classes of compounds: *Vinca* (or *Catharanthus*) alkaloids, epipodophyllotoxins, taxanes, and camptothecins. There are also a large number of phytochemicals subject to various phases of clinical trials, such as curcumin, epigallocatechin gallate (EGCG), soy isoflavones, etc. These compounds have shown anticancer effects both in vitro and in vivo. Some of them are discussed in the following section.

Resveratrol

Resveratrol is a polyphenol belonging to the stilbene class of phytochemicals. It is found in several plant species including grapes, peanuts, mulberries, cranberries, and other fruits [380, 381]. Resveratrol was found to be most abundant in the skin of grapes. It has been reported to block various cancer-related proliferative pathways making it a hopeful anticancer therapeutic candidate [382, 383]. A plant with considerably high content of resveratrol, *Polygonum cuspidatum*, is highly used in traditional Chinese medicine (TCM) to treat inflammation and cancer [384]. In 1997, resveratrol was first demonstrated to delay cancer initiation, promotion, and progression [385]. It is already used in clinical settings because of its antitumor cancer and chemopreventive activities [386]. Ongoing trials are investigating the possible effect of resveratrol on human cancers. Most clinical trials are testing the anticancer effects of resveratrol in CRC including NCT00256334, NCT00578396, NCT00920803, and NCT00433576. Two trials in GI cancers (NCT01476592) and thyroid cancers are assessing the effect of resveratrol on notch-1 signaling. The anticancer effect of resveratrol has also been investigated in leukemia, lymphoma, multiple myeloma, and prostate, breast, brain, and other nervous system cancers. In a bone cancer pain model, resveratrol was recently proposed to have palliative effects by blocking spinal glial activation and downregulating CX3CR1 [387].

Nuclear Factor-κB Pathway

Resveratrol has been shown to have anti-inflammatory and antitumor effects [388]. Resveratrol blocks cell proliferation and induces apoptosis in various cancer cell lines, such as breast, prostate, colon, and ovarian cancer cells [389]. The inhibitory effects of resveratrol on tumor growth have been attributed to its anti-inflammatory activity [389]. Aberrant regulation of NF-κB has been associated with cancer and autoimmune diseases. NF-κB is used by cells as a regulator of genes that control cell proliferation and cell survival. Many different types of human malignancies showed dysregulation of NF-κB. Resveratrol suppresses NF-κB activity mainly through blocking NF-κB inhibitor kinase (IKK) in murine and human macrophage cells along with downregulation of AP-1 [390, 391]. Resveratrol can downregulate NF-κB-induced gene products involved in inflammation, such as iNOS and COX-2, matrix metalloproteinase (MMP)-3, MMP-9, and vascular endothelial growth factor (VEGF) in macrophages and vari-

ous cancer cells [392, 393]. NF-κB-mediated transcriptional activity stimulated by EGF and TNF-α was effectively blocked by resveratrol in prostate cancer cell lines [394]. Resveratrol treatment in human multiple myeloma cell line inhibited proliferation by decreasing proliferative and antiapoptotic factors. The effect, which was mediated through suppression of NF-κB, potentiated the effects of bortezomib and thalidomide [395].

MAPK phosphatase 5 (MAPK5) is a potent inhibitor of cellular inflammatory responses because it can inhibit the enzymatic activation of MAPK, one of the upstream kinases that control the activation of NF-κB [396]. It has been reported that resveratrol could upregulate MAPK5 and block p38 pathway in prostate cancer cell lines [397]. Furthermore, resveratrol can inhibit NF-κB by blocking the upstream activator PKCδ and by activating the inhibitor SIRT1 [398].

Anti-inflammatory Implications: Focus on COX-2

Resveratrol is a potent COX suppressor, which has been confirmed in different in vivo and in vitro studies. Resveratrol can inhibit COX-2 activity through direct binding or suppression of transcription factors [399]. Resveratrol counteracts the proliferation of CRC and MCF-7 breast cancer cell line through affecting p53-COX-2 pathway. In vivo studies confirmed that resveratrol in dietary levels leads to a reduction in the formation of DMBA-induced mammary tumors through inhibition of COX-2-, MMP-9-, and NF-κB-mediated tumor cell proliferation [400].

In an interesting study, resveratrol was shown to prevent apoptosis induced in human leukemia K562 cells by H_20_2. In fact, resveratrol reversed the elevation of leukotriene B4 and prostaglandin E2 induced by H_20_2 challenge through inhibition of 5-lipoxygenase, COX, and peroxidase activity of prostaglandin H synthase [401].

Other Inflammatory Pathways

Resveratrol is also able to suppress the expression of hypoxia-inducible factor-1α (HIF-1α) through inhibition of MAPK and increased degradation of HIF-1α protein via the proteasome pathway. Resveratrol also suppressed VEGF through inhibition of HIF-1α [402, 403].

Recent studies have discussed the role of microRNAs (miRNAs) in mediating the anti-inflammatory effects of resveratrol. Resveratrol can decrease the secretion of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-8, and TNF-α), the expression of adhesion proteins including intercellular adhesion molecule (ICAM)-1, and the expression of leukocyte chemoattractants, such as MCP-1 [404]. Resveratrol suppressed TNF-α-induced signaling pathways both via NF-κB activation and by increasing transcriptional activity of p65 [405]. In addition, resveratrol induced the expression of Egr-1 from its chromosomal locus. Egr-1 has demonstrated antitumor effects upon experimental increase in TNF-α [406]. The control of transgenic expression via activation of Egr-1 promoter by resveratrol may sensitize cancer cells, expanding the use of adenovector Ad.Egr-TNF in patients resistant to radiation or chemotherapy, suggesting a new means for development of inducible gene treatments [406]. In prostate cancer cell line, resveratrol increased the production of ROS and expression of pro-apoptotic factors including TRAIL [383]. In a mouse model with prostate cancer, resveratrol significantly reduced cell proliferation and the expression of growth factors and their receptors [383].

In human colon cancer cells resistant to the cytotoxic effect of resveratrol, resveratrol was able to sensitize tumor cells to TNF, anti-CD95 antibody, and TRAIL-mediated apoptosis and led to activation of a caspase-dependent death pathway [407]. Indeed, resveratrol sensitized lung cell lines to TNF-induced apoptosis by modifying sirtuin effect, and this activity is consistent with its ability to induce activity of Sirt1, a known NF-κB transcription repressor. Polyphenols can augment TRAIL expression in gastric cancer cell lines and are able to increase TRAIL-mediated apoptosis in various cancer types such as human melanoma, prostate carcinoma, pancreatic cancer, malignant glioma, prostate carcinoma, hepatocellular carcinoma, gastric carcinoma, neuroblastoma cells,

Burkitt's lymphoma, ovarian cancer cells, renal cancer cells, and colon cancer cells [408].

Resveratrol inhibited epithelial-mesenchymal transition (EMT) of pancreatic cancer cells by downregulating both the PI3K/AKT/NF-κB pathway and the EMT-related gene expression (E-cadherin, N-cadherin, vimentin, MMP-2, and MMP-9), which are essential for cancer cell motility and metastasis [409, 410]. In human pancreatic cancer cell, resveratrol treatment induced transcriptional upregulation of macrophage inhibitory cytokine-1 (MIC-1), which has antitumor activity [411]. Resveratrol is capable of blocking mediators of metastasis including lysophosphatidic acid (LPA), transforming growth factor (TGF), and focal adhesion kinase (FAK) in cancer cells like ovarian carcinoma cell. LPA induces the expression of HIF-1α and VEGF and thereby promotes cell migration [403]. Additionally, resveratrol can inhibit TGF-β1 and so cause inhibition of cell adhesion, migration, and invasion of lung cancer cells in A549 lung cancer cells [412, 413]. Resveratrol could diminish cell proliferation by influencing autocrine growth modulator pathways in breast cancer cells. For instance, it can increase the expression of the growth inhibitor TGF-ß2 without affecting the expression of TGF-ß1 and TGF-ß3 [414, 415]. Resveratrol may be used to modify the immunological reaction in tumor microenvironment, including inhibition of T-cell proliferation, reduction of IFN-γ and IL-4 secretion, downregulation of B-cell proliferation and therefore production of IgG1 and IgG2a isotypes, and suppression of CD28 expression on $CD4^+$ T-cells and CD80 on macrophages [416].

Other possible antitumor effects from an immunological viewpoint include downregulation of MHC class I and II molecules; induction of tolerogenic DC phenotype; downregulation of the ability of bone marrow (BM)-derived DC to produce IL-12 p70 [417]; increasing the production of TNF-α, IL-12, and IL-1β in response to LPS stimulation; enhancing the secretion of IL-10; suppression of mucosal and systemic $CXCR3^-$-expressing effector T-cells and inflammatory cytokines in the colon [418]; and inhibition of the suppressive activity of FoxP3-expressing T_{REG} cells among $CD4^+CD25^+$ cells [416, 419–423].

Low-dose resveratrol was able to enhance cell-mediated immune responses by promoting T_H1 cytokine production, macrophage function, and also APC-induced IL-12 and IFN-γ production [424]. Resveratrol treatment downregulated the frequency of T_{REG} cells in EG7-bearing C57BL/6 mice. In addition, both $CD4^+CD25^+FoxP3^+$ to $CD4^+CD25^+$ cell ratio and $CD4^+CD25^+$ to $CD4^+$ cell ratio were reduced concurrently by resveratrol in a dose-dependent manner [425]. Resveratrol has been mostly investigated as an adjuvant agent combined with conventional chemotherapeutics to prevent or reduce the risk of multidrug resistance. Resveratrol strengthened the antitumor effect of 5-fluorouracil (5-FU) on CRC cells, thereby enhancing chemosensitization and reducing drug resistance [426]. For example, resveratrol sensitized various human cancer cell lines to chemotherapeutic agents such as doxorubicin, cytarabine, actinomycin D, Taxol, and methotrexate by suppressing the expression of survivin and enhancing apoptosis. The mechanism by which resveratrol chemosensitizes cancer cells includes inhibition of tumor cell proliferation, metastasis, and angiogenesis and induction of tumor cell apoptosis through inhibition of related signaling pathways, such as SIRT1, signal transducers and activators of transcription 3 (STAT3), Hh, AMPK/YAP, PTEN/PI3K/AKT, and NF-κB [427–430]. Moreover, NF-κB activation could upregulate the levels of some antiapoptotic genes, including TNF receptor-associated factor 1 (TRAF1) and TRAF2 [431]. Administration of resveratrol in $IL\text{-}10^{-/-}$ mice induced immunosuppressive $CD11b^+Gr^-1^+$ MDSCs in the colon. The stimulation of immunosuppressive $CD11b^+Gr^-1^+$ cells by resveratrol during colitis is distinctive and offers a novel mode of anti-inflammatory action of resveratrol [418].

AhR and Nrf2 as Inflammation-Environment-Diet Molecular Crossroads

AhR functions as a modulator of immunity (inflammation) and reaction to xenobiotics on one hand and acts as a mediator of effect of res-

veratrol on the other hand. Moreover, it is interesting to mention that the effect of resveratrol is frequently associated with upregulation or activation of Nrf2 [432, 433]. Resveratrol also augments the activation of nuclear factor E2-related factor-2 (Nrf2), which is followed by activation of antioxidant response element (ARE). Resveratrol has been reported to increase the expression of heme oxygenase-1 (HO-1) via Nrf2 activation in PC12 cells. In leukemia K562 cells, resveratrol increased NQO1 expression and stimulated Nrf2/Keap1/ARE binding to NQO1 promoter [434]. It also restored glutathione levels in human lung cancer A549 cells treated with cigarette smoke extracts by increasing the Nrf2-induced GCL expression [435]. There are some dietary AhR antagonists such as genistein, kaempferol, and EGCG. One recent agonist of AhR causes a number of anti-inflammatory responses in vitro and in vivo [436, 437]. Resveratrol assists Nrf2 and AhR in maintaining homeostasis against inflammatory insults, which may be involved in tumorigenesis. For instance, resveratrol caused inhibition of TCDD-induced recruitment of AhR and ARNT to the CYP1A1/CYP1A2 and CYP1A1/CYP1B1 promoter in hepatic cancer (HepG2) and breast cancer cell (MCF-7), respectively [438]. Therefore, resveratrol could modulate the activity of some cytochrome P450 enzymes and so act as chemopreventive compound by limiting activation of pro-carcinogens.

Immune Surveillance

Downregulation of tumor immunosurveillance involves resistance to apoptosis, production of immunosuppressive cytokines, and reduced expression of MHC class I antigens. Particularly, macrophages inhibit or increase the growth and spread of cancer based on their activation state. Synthetic resveratrol analog, HS-1793, significantly increased IFN-γ-secreting cells in splenocytes and also decreased $CD206^+$ macrophage infiltration [439]. The local augmentation of IFN-γ modified the status of tumor-associated macrophages (TAMs) associated with the cancer microenvironment that occurred coincident with increased levels of pro-inflammatory and immunostimulatory cytokines (CD206, CD204, IL-10, TGF-β, EGF, and MMP-9) and decreased levels of IL-6 and immunosuppressive and tumor progressive mediators [439]. However, further studies are necessary to clarify the mechanism of action of resveratrol. Oral resveratrol significantly improved survival of lymphocytic leukemia L1210 cell-bearing mice through normalization of CD4/CD8 ratios and enhancement of NK cell activities and antisheep RBC titers. Furthermore, resveratrol suppressed cellular content, release, and mRNA expression of IL-6 [440].

CD95 Signaling Pathway

The Fas receptor (FasR), also known as CD95, Apo-1, and tumor necrosis factor receptor superfamily member 6 (TNFRSf6), leads to apoptosis. Resveratrol induces tumor cell death by modifying the levels of Fas and its ligand, FasL [441–443]. Earlier studies have reported this effect in leukemia cell lines [441] and colon [442] and breast carcinoma cells [443]. A study in multiple myeloma and T-cell leukemia cells emphasized the role of Fas/CD95 signaling in lipid rafts in anti-myeloma and anti-leukemia chemotherapy [444]. Using leukemia lines derived from patients with malignancies pro-B t(4;l1), pre-B, and T-cell ALL, it has been demonstrated that resveratrol could induce extensive apoptotic cell death not only in CD95-sensitive leukemia lines but also in B-lineage leukemic cells that are resistant to CD95 signaling [445]. Altogether, the CD95-CD95L system and its chemotherapeutic and chemopreventive potential are interesting enough to be considered in anticancer drugs [446].

Resveratrol and Its Interplay with NK Cells

Direct influence of resveratrol on NK cells and their killing ability on different levels has been reported in previous studies. Resveratrol exerts concurrent effects on NK cells and other immune cells like $CD8^+$ and $CD4^+$ T-cells [447]. The killing ability of NK cells against human immortalized myelogenous leukemia K562 cells was increased after resveratrol treatment. Furthermore, a dose-related inhibition of lytic activity was reported at high concentrations of

resveratrol. Another study reported blocking of viability and enhanced apoptosis of NK cells upon incubation with high concentrations of resveratrol, whereas low concentrations of resveratrol resulted in upregulation of NKG2D and IFN-γ and increased killing of leukemia K562 target cells by NK cells [448]. Higher vulnerability of human lymphoblastoid T-cells (Jurkat cells) to cytotoxic effect of resveratrol also has been reported [449, 450]. Resveratrol in NK-92 cells increased the expression of perforin and phosphorylation of ERK-1/ ERK-2 and JNK, which are known to contribute NKG2D-mediated cytotoxicity [450]. Intragastric administration of resveratrol enhanced the killing ability of isolated spleen NK cells against mouse 51Cr-labeled lymphoma [451].

Furthermore, resveratrol increased the expression of NKG2D ligands on human promyeloblastic leukemia KG-1a cells, thus offering two mechanisms to potentiate cytokine-induced killer cells (CIK, a mixed phenotype between T-cells and NK cells) [452]. Stimulation of KG-1a cells susceptible to CIK-mediated cytolysis occurs via an increase in cell surface expression of NKG2D ligands and receptor DR4 and also via suppression of DcR1 along with activation of the TRAIL pathway [452]. Resveratrol may modify this axis, thereby promoting tumor surveillance by the innate immune system. Resveratrol is further capable of sensitizing cells of various cancer types, including neuroblastoma, medulloblastoma, glioblastoma, melanoma, T-cell leukemia, and pancreatic, breast, and colon cancer, to TRAIL-induced apoptotic cell death [453, 454]. In essence, resveratrol can upregulate the expression of receptors DR4 and DR5 in human prostate cells [455], thus enhancing TRAIL sensitivity and possibly facilitating NK cell-mediated killing activity. Resveratrol also considerably increased CD95L expression on HL-60 human leukemia cells and on T47D breast carcinoma cells [446], which would further help in NK cell-mediated apoptosis. Resveratrol has another therapeutic potential in defeating aggressive NK cell leukemias and lymphomas through inhibition of constitutively active signal transducers and activators of transcription 3 (STAT3) signaling [456].

Possible Interaction with T_{REG}

Resveratrol is also able to decrease the cell number and function of immune T_{REG} cells. High-dose IL-2 (HDIL-2) led to T_{REG} expansion, but it was inhibited by resveratrol which could abrogate the toxic effects of HDIL-2 on endothelial cells [457]. Resveratrol was also involved in suppression of TGF-β secretion from the spleen of tumor-bearing mice and concurrent increase in IFN-γ expression in $CD8^+$ T-cells, together resulting in immune stimulation [423]. Despite its immunostimulatory activity, IFN-γ is also reported to induce T-cell inhibitory molecule IDO in many cell types, including APCs [458]. Resveratrol can inhibit IFN-γ-induced IDO expression in bone marrow-derived dendritic cells (BMDCs) [459]. Resveratrol-mediated inhibition of EG7 thymoma tumor growth was dependent on IDO through inhibition of the Jak/Stat pathway and protein kinase C-δ (PKCδ), which both need IFN-γ-mediated IDO expression [460]. Resveratrol combined with thymoquinone was reported to decrease tumor size and increase serum levels of INF-γ in breast cancer tumor-bearing mice [461].

Regulatory B-Cells

The most fascinating antitumor immune mechanism of action of resveratrol is through inhibition of tumor-induced regulatory B-cells (tBregs), which inhibit breast cancer metastasis [462, 463]. Low concentrations of resveratrol significantly decreased tBregs (defined as CD25+ $CD81_{high}$ cells within the CD19+ population) and Treg populations in mice. It must be emphasized that resveratrol had no effect on MDSCs in the tumor models [462, 463].

Modulation of Mucosal Integrity: Implication of MUC2 and MUC1

Oral administration of resveratrol activated the expression of MUC2 and inhibited the expression of MUC1 through modification of the enzymes that initiate *o*-glycosylation of mucin in 1,2-dimethylhydrazine (DMH)-treated rats. Therefore, resveratrol assists in maintaining integrity of the colon [464] through modification of enzymes that initiate *o*-glycosylation of mucin [465].

Curcumin

Curcumin is the active polyphenol derived from the *Curcuma longa* plant, which is also known as turmeric. Two curcuminoids, demethoxycurcumin and bisdemethoxycurcumin, exhibit antiproliferative activity on various cancer cells [466–468]. Curcumin has been reported to be effective as a therapeutic and preventive agent for cancer of the colorectum, liver, lung, pancreas, breast, ovary, uterine, bladder, prostate, kidney, and brain, non-Hodgkin lymphoma, and leukemia [469–471]. It can exert effective immune responses and cytotoxic activity on different cancer cell lines, such as YAC-1 murine lymphoma, human HL-60 leukemia, and MDAMB breast carcinomas [472]. In vivo studies have shown immunostimulatory effects of curcumin [472, 473].

Mechanisms of Action of Curcumin: A Role for NF-κB

Inflammation has been implicated in the different steps of tumorigenesis, including induction, survival, proliferation, invasion, and metastasis. Primary studies described curcumin as an effective modulator of inflammation [474]. The direct effect of curcumin on inflammation has been attributed to inhibition of NF-κB signaling. NF-κB is a transcription factor that controls the expression of several genes involved in growth, inflammation, carcinogenesis, and apoptosis [475]. Curcumin can inhibit this pathway through downregulation of the activation of IκBα kinase (IKK), phosphorylation and degradation of IκBα, and phosphorylation and nuclear translocation of the p65 subunit [476, 477] in several cancer and premalignant cell types [478, 479]. The results were confirmed in cells isolated from patients with multiple myeloma [480] and advanced pancreatic cancer [481]. As NF-κB regulates several pathways like MMP, inhibition of NF-κB leads to downregulation of molecular events implicated in other signaling pathways and thus offers different opportunities for prevention and treatment [482] as indicated in several studies [483–485]. For instance, curcumin suppresses the production of CXC chemokines through inhibition of the NF-κB pathway [486]. In addition, the expression of multiple NF-κB-regulated gene products, including IL-6, IL-8, MMP-9, COX-2, and CCL2, was reduced with curcumin. Furthermore, curcumin also affects other inflammatory markers and subsequent tumor promotion [474], such as inflammatory cytokines (TNFα, IL-1, IL-6, and IL-8) [487, 488], inflammatory transcription factors (STATs), and inflammatory enzymes (COX-2, 5-lipoxygenase (LOX)) [489]. Curcumin can inhibit different invasion, cell adhesion, and extracellular matrix molecules, such as matrix metalloproteinase, CCRX4, COX-2, ELAM1, and ECAM1 [490].

Curcumin can inhibit iNOS induction and scavenge NO radicals in breast cancer cells in the promotion phase of carcinogenesis [491, 492]. TNF-α is a direct stimulator of aerobic glycolysis in malignant breast epithelial cell lines, and interestingly curcumin could reverse this effect of TNF-α [493].

Curcumin strongly prevents the generation of hematogenous metastases through suppression of the expression of NF-κB/activator protein-1 (AP-1)-dependent MMP, Egr-1, [494], and other genes involved in cell adhesion (chemokines, TNF, and Cox-2) [495, 496]. On the other hand, inhibition of NF-κB reduced the expression of prometastatic chemokine (C-X-C motif) ligand (CXCL) 1 and 2, which, in turn, decreased the expression of chemotactic receptor CXCR4 along with other prometastatic genes [486]. Decreased expression of matrix metalloproteinases, ICAM-1, and CXCR4 along with suppressed cell migration and invasion has been reported in breast cancer cell line [497].

Effect of Curcumin on Matrix Metalloproteinase-9 (MMP-9)

MMPs have been considered as one of the important molecules assisting tumor cells during metastasis [498, 499]. MMP-9 shows a major role in the breakdown of extracellular matrix in disease processes such as tumor metastasis [500]. However, curcumin shows a vital role in the inhibition of MMP-9 activities and cell invasion through downregulating NF-κB [501].

Restoration of $CD4^+$ and $CD8^+$ T-Cell Populations and Increased T_H1-Type Response

Curcumin could efficiently restore $CD4^+$ and $CD8^+$ T-cell populations in the tumor microenvironment and prevent depletion of central memory and effector memory T-cells in peripheral circulating blood and lymph nodes and at the tumor site. In this manner, curcumin can drive T_H2 cytokine response toward a T_H1-type response [502, 503]. However, results regarding this point are not consistent. These contradicting reports suggest that curcumin may be implicated in complex signaling pathways, leading to an enhanced antitumor immunity. Curcumin is able to reverse the decrease in the levels of T_H1 cytokines such as IFN-γ and the increase in T_H2 cytokines such as IL-4 during cancer progression. Although some studies suggest different outcomes in which curcumin favors a T_H2-type response, there are studies reporting that curcumin supports cancer regression by restoring T_H1 immune responses [504, 505]. The elevated population of tumor-infiltrating lymphocytes leads to increased tumor cell killing. A delayed NK cell cytotoxic response and a simultaneous increase in IL-12 secretion in the serum of treated mice were reported after curcumin treatment [472]. Curcumin might prevent T-cell depletion by inhibiting secretion of suppressive molecule PGE-2 by tumor cells [506]. PGE-2 inhibits expression of the cytokine receptor gamma chain (γc) in T-cells, which causes deactivation of the Jak/Stat pathway and reduces expression of pro-survival protein Bcl-2 in T-cells. Curcumin through inhibition of PGE-2 would restore γc and Bcl-2 expression in T-cells and so support T-cell survival and differentiation [507].

It has been reported that curcumin arrests maturation of DCs and stimulates a tolerogenic phenotype that next promotes functional $FoxP3^+$ T_{REG} cells. It has been shown that DCs generated in the presence of curcumin had minimal CD83 expression, suppressed levels of CD80 and CD86, and reduced expression of both MHC class II and CD40 in comparison with those DCs that were differentiated in the absence of curcumin. Curcumin enabled arrested maturation of DCs and induced a tolerogenic phenotype [473, 503, 508, 509]. An increase in the generation of $CD4^+CD25_{high}CD127_{low}$ $FoxP3^+$ T_{REG} cells that exert suppressive functions on naïve syngeneic T-cells has also been observed with curcumin treatment [508]. Curcumin prevented loss of effector and memory T-cells, extended central memory T-cell (TCM)/effector memory T-cell (TEM) populations, reversed T_H2 immune response, and attenuated tumor-induced inhibition of T-cell proliferation in tumor-bearing hosts [510].

Reduction of T_{REG} Cell Population

$CD4^+CD25^+FOXP3^+$ T_{REG} cells play an important part in the tumor immune evasion process. Progression of tumor coincides with an elevation in T_{REG} cells, which secrete immunosuppressive cytokines like TGF-β and IL-10 and express the high-affinity IL-2 receptor CD25, which sequesters IL-2 from the tumor milieu. It must be noted that IL-2 is necessary for proliferation of other T-cells, and so its reduction leads to effector T-cell apoptosis [511, 512].

Curcumin is able to block IL-2 signaling by decreasing accessible IL-2 and high-affinity IL-2R, as well as interfering with IL-2R signaling. Curcumin has also been demonstrated to block IL-2-induced phosphorylation of STAT5A and Janus kinase (JAK), but not JAK1, suggesting inhibition of critical proximal events in IL-2R signaling [513].

Curcumin can efficiently decrease T_{REG} cell number and the levels of IL-10 and TGF-β [514]. Other studies also reported similar results, suggesting that treatment of $CD4^+CD25^+$ T_{REG} cells with curcumin decreased their immunosuppressive activity [472, 515]. FOXP3 and CTLA-4 are essential for T_{REG} function [516]. It has been shown that curcumin can reduce the expression of CTLA-4 and FOXP3, two key transcription factors that are involved in regulating transcriptional program of T_{REG} cells and are necessary for development and function of T_{REG} [516]. Curcumin inhibited T_{REG} function by blocking cell-cell contact [514].

Increased oxidative stress in tumor inhibits NF-κB activity in thymic T-cells, which makes

T-cells vulnerable to apoptosis by TNF-α secreted from tumor cells [517, 518]. Curcumin through inhibition of oxidative stress and reduction of surface expression of TNF-α receptor (TNFR1) on thymic T-cells of tumor-bearing mice [517] prevents reduction of NF-κB activity in thymic T-cells.

Curcumin treatment can inhibit the tumor suppressor indoleamine-2,3-dioxygenase (IDO) as well as the immunosuppressive cytokine TGF-β, thereby promoting T-cell cytotoxic activity [519]. IDO exerts its immune suppressive effect by catalyzing tryptophan, which is necessary for T-cell proliferation [520].

Reduced T-Cell Apoptosis

Prolonged injections of curcumin maintained levels of T_H1 cytokines, NK cell cytotoxic activity, and production of ROS and NO by macrophages [472]. Tumor-bearing mice treated with curcumin showed improvement in immune cell numbers and tumor regression, consistent with inhibition of apoptosis in thymocytes and splenocytes [502]. Curcumin reduced T-cell apoptosis in tumor-bearing mice through activation of the JAK3-STAT5a pathway in T-cells and subsequent restoration of BCL-2 levels [506]. Inhibition of tumor-induced thymic atrophy by restoring the activity of NF-κB pathway also has been reported after curcumin treatment [517]. Eventually, although low dose of curcumin stimulated effective antitumor response by escalating $CD8^+$ cytotoxic T-cells and IFN-γ production, higher dose of curcumin was harmful for T-cells [473].

STAT Pathway

STATs modify tumor-promoting inflammation via collaboration of other transcription factors [474, 521]. Curcumin inhibits the expression of STATs, especially nuclear STAT3, STAT5a, and STAT5b in human chronic K562 leukemia cells. When used as a pretreatment, curcumin inhibited IFN-γ-induced phosphorylation of nuclear STAT1 and STAT3 [522, 523] in human K562 leukemia cells and STAT1 in human lung A549 carcinoma and melanoma cells [524]. Following treatment with curcumin and its analogs such as GO-Y030 [525], FLLL1, and FLLL12 [526], similar downregulation of STAT3 activation was also observed in Hodgkin's lymphoma [483], T-cell leukemia [527], head and neck squamous cell carcinoma [528], multiple myeloma cells [529], and $CD138^+$ cells derived from multiple myeloma patients [480].

Curcumin alone or in combination with epigallocatechin gallate (EGCG) blocked STAT3 phosphorylation and undermined the interaction between STAT3 and NF-κB through suppression of CD44 expression, together diminishing breast cancer stem cells (bCSCs) population [530, 531].

COX-2

Curcumin is an effective inhibitor of COX-2 in several cancer types [532–535]. Moreover, curcumin can inhibit COX-2 expression in PBMCs of patients with pancreatic cancer [481] and on oral premalignant cells [476]. Furthermore, fluorocurcumin, a curcumin analog, has been reported to suppress NF-κB and PGE-2, and so it was suggested to be a potential agent against COX-2-overexpressing tumors [536]. Curcumin downregulates the expression of EGFR in pancreatic and lung adenocarcinoma expressing COX-2 [537] through inhibition of ligand-induced activation of EGFR [538] or through decreasing the transcriptional activity of Egr-1.

Synergy with Drugs

Several studies investigated the potential synergistic activity of curcumin in combination with conventional chemotherapeutic agents. Curcumin combined with omega-3 fatty acid could suppress the expression and activity of iNOS, COX-2, and 5-LOX and upregulation of p21 [534] and therefore prevent or even treat pancreatic tumor xenografts [534]. Curcumin would potentiate the effect of paclitaxel-mediated chemotherapy in advanced breast cancer in vitro and in vivo. This effect has been attributed to suppression of NF-κB and serine/threonine Akt pathways, COX-2, and MMP-9 [539, 540]. Reduction of COX-2 is also reported in human colon cancer HT-29 cell lines treated with curcumin combined with 5-FU [541]. Although prostate and breast cancer cells (DU145, PC-3, and LNCaP) are typically resistant to TRAIL-induced apoptosis, they can be

sensitized with curcumin. This mixture stimulates inhibition of active NF-κB and other pathways that also were confirmed by preclinical studies performed in PC-3- and TRAIL-resistant LNCaP xenografts [542–545].

Interleukins

M2 macrophages and T_{REG} cells are two main leukocytes that secrete the anti-inflammatory cytokine IL-10 [546]. M2 macrophages play a critical role in tumor progression and development consistent with increased IL-10 concentrations in various solid tumors. M1 macrophages produce IL-12, an antitumor chemokine. So, the IL-10/IL-12 ratio might predict tumor progression [547]. IL-10 could inhibit several components of immunity, including co-stimulatory and adhesion molecules (CD86 and CD54) that induce an inflammatory response in macrophages [548] and cytokines such as IL-12, IL-23, IL-1β, and TNF-α that are involved in inflammatory immune response [548–550]. IL-10 enhances the activation and proliferation of B-cells and antibody production. Maintaining the T_H1/T_H2 balance is one of the important facets of immunomodulatory action of IL-10. IL-10 has potential anticancer effects which may be mediated through reductions in the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 that play important roles in neovascularization as well [551, 552].

Curcumin can increase the frequency of M1 macrophages while decreasing the frequency of M2 macrophages, resulting in a decrease in the expression of STAT3, IL-10, and arginase-1 in mice with metastatic breast cancer. Through reduction in IL-10 levels, curcumin can also block Janus kinase-STAT signaling and increase tumor cell apoptosis [547].

Curcumin can act as an antitumor agent through prevention of tumor-induced T-cell depletion by increasing the production of IFN-γ, an important T_H1 cytokine for the production and function of peripheral T-cells, and IL-2, which is crucial for differentiation of cytotoxic T-lymphocytes. Antitumor activity of curcumin could also be due to the restoration of activated/effector $CD4^+$ and $CD8^+$ T-cells, induction of tumor-infiltrating lymphocytes (TILs), and upregulation of IFN-γ expression. Curcumin also reduces the levels of TGF-β and IL-10 in T_{REG} cells and decreases the number of T_{REG} cells in the tumor microenvironment [503].

IL-8 was overexpressed in ER-negative cancer cells and showed a potential correlation with tumor progression and invasiveness. Overexpression of IL-8 is linked to progression and metastasis of cancer cells in the colon [553]. Treatment of colon cancer cells with curcumin inhibited neurotensin-induced gene expression and protein secretion of IL-8, thereby preventing migration of cancer cells [554]. Curcumin also reduced the expression of IL-8 in human pancreatic cancer cell line [555].

Exosomes and Immune Suppression in Cancer

Exosomes are small particles that are released from normal and neoplastic cells and are present in serum and other bodily fluids. Exosomes have various molecules including signal peptides, mRNA, and microRNA. Tumors also secrete exosomes which are immune suppressive bodies containing a distinct set of proteins that can affect the immune system. In cancer, signaling via exosomes affects the immune system through inhibition of T-cell and NK cell functions and an increase in the number and/or activity of immune suppressor cells, including myeloid-derived suppressor cells (MDSCs), T_{REG} cells, and $CD116^+$ HLA-DR−/low cells [556]. Curcumin reduces the inhibitory effects of exosomes on NK cytotoxicity [557]. Of note, curcumin can reverse the tumor exosome-mediated inhibition of NK cell function via the ubiquitin-proteasome pathway [558].

Green Tea and Catechins

Several epidemiological and experimental studies have reported a negative correlation between green tea and development of cancers of the bladder, cervix, breast, esophagus, colorectum, stomach, lung, liver, ovaries, oropharynx, pancreas, prostate, and skin [559]. The health benefits of

green tea could be mostly attributed to catechins, including catechin (C), epicatechin (EC), epigallocatechin (EGC), and epicatechin gallate (EGCG).

Transcription Factors

EGCG has been found to suppress the expression and/or activity of many transcription factors, such as HIF-1α, nuclear STAT1 and STAT3, NF-κB, and AP-1. In addition, different MMPs, including MMP-2, MMP-9, and MMP-14/MT1-MMP, have been downregulated by EGCG [559]. EGCG has been reported to block angiogenesis and decrease xenograft tumor growth via inhibition of IGF-1 through downregulating the protein expression of HIF-1α and VEGF in A549 lung cancer cells [560, 561] and via inhibition of HIF-1α-dependent expression of VEGF, IL-8, and CD31 in other lung NCI-H460 cell lines [562]. EGCG blocked xenograft angiogenesis and tumor growth in gastric cancer cell line BGC-823 [563]. EGCG is also able to inhibit IL-6-induced angiogenesis via inhibition of VEGF expression through downregulating Stat3 activity in human gastric carcinoma AGS cells and SGC-7901 cancer cells [564, 565]. In HeLa cervical cancer cell line, EGCG inhibited cell proliferation and invasion through suppression of MMP-9 gene expression and upregulation of TIMP-1 gene expression [566]. In SW837 CRC cell line, EGCG inhibited tumor growth by downregulating HIF-1α and several major growth factors [567]. In T-24 bladder cancer cell line and SW620 cell line, EGCG inhibited cell adhesion, migration, and invasion through suppression of MMP-9 expression via inhibition of NF-κB signaling pathway [568]. In esophageal TE-8 and SKGT-4 cancer cells, EGCG reduced cell invasion through lessening p-ERK1/p-ERK2, c-Jun, and COX-2 [569].

Overexpression of the human epidermal growth factor receptor-2 (HER2/neu) is linked to poor prognosis in various types of cancer. EGCG blocks activation of these receptors by inhibiting STAT3 and NF-κB activation. EGCG and Polyphenon E (PolyE) have been shown to decrease transcriptional activity of AP-1 and NF-κB promoters and inhibit COX-2 transcription and PGE-2 production in CRC cell lines [570].

Effect of Green Tea on Nuclear Transcription Factor NF-κB

EGCG has been reported to inhibit the activation of NF-κB in H891 human HNSCC cells, MDA-MB-231 human breast cancer cells, PC-9 human lung cancer cells, human colon cancer cells, A431 epidermoid carcinoma cells, and H891 head and neck cancer cells. EGCG decreased lipopolysaccharide (LPS)-induced TNF production in the RAW 264.7 macrophage cell line [571]. Treatment with EGCG and PolyE reduced the levels of inflammatory cytokines, such as TNF-α, in the colon epithelium and also inhibited inflammation-related colon carcinogenesis induced by AOM and DSS injection in a mouse colon cancer model [572].

Regulation of the NF-κB pathway may play a critical role in mediating chemopreventive properties of catechin in prostate cancer cells. Catechin treatment regulates NF-κB gene expression through accumulation of IκBα, repression of NF-κB phosphorylation [573], reduction in IKKα expression, inhibition of IKK activity [574] and proteasome and caspase cleavage of the p65 subunit [575], and reduction in other signaling factors, including RANK and NIK [573]. NF-κB target genes involved in carcinogenesis, including Bcl-2, Bcl-xL, survivin, MMPs, VEGF, uPA, and iNOS [576–578], are also decreased by catechin treatment. Thus, one of the probable mechanisms by which EGCG can exert antitumor effects is through suppression of the NF-κB signaling pathway.

EGCG treatment resulted in decreased COX-2 promoter activity through inhibition of NF-κB activation [579]. AP-1 serves as another potential target for anticancer effects of EGCG [580]. EGCG has been demonstrated to interfere with AP-1-induced transcriptional activity through inhibition of a JNK-dependent pathway [581].

Effect of Green Tea Catechins on Cyclooxygenase and Lipoxygenase

EGCG has been reported to inhibit mitogen-induced COX-2 expression in androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells [582]. Pretreatment with green tea catechins inhibited

COX-2 expression induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin and reduced COX-2 expression in the SW837 human CRC cell line, colon epithelium, and LPS-induced macrophages. It has been shown that EGCG decreases the activity of COX-2 after IL-1A stimulation of human chondrocytes [583]. Recent cancer research suggests that development of compounds, which can inhibit COX-2 expression preferably without affecting COX-1, is a hopeful approach for cancer chemoprevention. The inhibition of NF-κB is suggested as a possible mechanism for inhibition of COX-2 expression. EGCG, EGC, and ECG from green tea and theaflavins from black tea have been reported to inhibit lipoxygenase (LOX)-dependent activity by 30–75% [584]. Consumption of green tea and dietary fat modulates 5-lipoxygenase-dependent pathway of arachidonic acid metabolism throughout AOM-induced colon carcinogenesis [585].

Effect of Green Tea on AP-1 Transcription Factor

AP- 1 is another transcription factor including Jun and Fos protein families that regulates expression of gene associated with apoptosis and proliferation. AP-1 has been implicated in cancer development and progression. AP-1 is induced by TNF and IL-1 as well as by a variety of environmental stimulators like UV radiation. Theaflavins and EGCG inhibited ultraviolet B (UVB)-induced AP-1 activation [586] and AP-1-dependent transcriptional activity and DNA binding activity [587, 588]. A previous study in JB6 mouse epidermal cell line demonstrated that EGCG treatment inhibits AP-1 activation and cell transformation and Ras-activated AP-1 activity in the H-ras-transformed cells. EGCG inhibits AP-1 activity through inhibition of JNK but not ERK activation [586]. EGCG or PolyE treatment causes inhibition of AP-1 and NF-κB luciferase reporter activity in the HT29 human colon cancer cell line. These findings indicate that inhibition of the NF-κB and/or AP-1 pathways is a possible mechanism underlying anticancer effects of green tea catechins [589, 590].

Effect of Green Tea on STAT3

EGCG inhibited phosphorylation of EGFR, Stat3, and ERK proteins in human HNSCC cell lines such as YCU-N861 and YCU-H891 [591]. Inhibition of activation of the EGFR, Stat3, and Akt by EGCG treatment has been shown in YCU-H891 HNSCC and MDA-MB-231 breast carcinoma cell lines [592].

EGCG-induced increase in IFN-γ secretion in a previous study has been attributed to an increase in NK and NK T-cell numbers that could be due to induced STAT1 activity. A previous clinical trial on 20 patients with stage IV cancer with a special regime containing soy extract reported increased cytotoxic activity of NK cells and TNF-α secretion [593]. An aggressive combination of immunoactive nutraceuticals was efficient in significantly increasing NK function [593].

Inflammatory Factors

Different studies reported that EGCG is able to inhibit the expression of various inflammatory factors in tumor cells including inflammatory cytokines (IL-8), inflammatory growth factors (insulin-like growth factor 1 (IGF-1) and VEGF), and inflammatory mediators (COX-2 and iNOS). In addition, it can inhibit the expression of chemokines, such as the colony-stimulating factor 1 (CSF-1) and C-C motif chemokine ligand 2 (CCL2). Therefore, targeting different inflammatory factors might play an important role in EGCG-mediated cancer inhibition [559, 594–597].

Modulation of Antitumor Immunity

Green tea has been reported to enhance humoral and cell-mediated immunity, resulting in reduced risk of certain cancers [579]. IDO, an immune regulatory enzyme, is associated with tumor immune escape. EGCG has been reported to downregulate the expression of IDO in human oral and colorectal cancer cells by inhibition of STAT1 function [579], concurrent with increased antitumor immunity. This indicates that EGCG can be a potential regulator of tumor immunity [598, 599].

Myeloid-Derived Suppressor Cells

MDSCs contribute to the negative regulation of immune responses. MDSCs downregulate T-cell

function through generation of arginase, NO, ROS, and peroxynitrate. However, in the tumor microenvironment, MDSCs are able to differentiate into tumor-associated macrophages (TAMs) and express arginase and iNOS and suppress generation of ROS [600, 601]. Besides antigens and co-stimulation, cytokines are required for T-cell activation, proliferation, and maintenance. Recent studies have shown that cytokines (IL-12 or IFN-γ) released by DCs or other APCs can act as the third signal that is responsible for activation, expansion, and appropriate production of effector and memory T-cells [602]. However, the tumor microenvironment cannot supply such inflammatory signals, leading to inappropriate activation of DCs. Furthermore, tumors produce immunosuppressive cytokines such as IL-10 and TGF-β and also increase T_{REG} cell number, which both further dampen proper DC activation [600].

Myeloid cells hamper the function of T- and NK cells. It is well known that tumor-induced T_{REG} cells blunt NK and $CD4^+/CD8^+$ T-cell-mediated immune responses. PolyE is able to promote the differentiation of MDSCs into more mature neutrophil-like cells with hypersegmented nuclei [603]. These cells are unable to inhibit the secretion of IFN-γ from $CD3^+$ splenocytes in vitro. MDSCs were less infiltrated into the neuroblastomas of mice drinking PolyE in comparison with control group. This confirms the hypothesis that catechins hinder the migration of myeloid cells to the tumor site. MDSCs interfere with the antitumor activity of $CD8^+$ T-cells. Intriguingly, another study has reported that EGCG enhances $CD8^+$ T-cell-mediated antitumor immunity as obtained by DNA vaccination. Depletion of immunosuppressive T_{REG} cells by means of a CD4-specific antibody decreases the growth of neuroblastomas in A/J mice [604]. In another report, depletion of $CD4^+$ cells failed to modify tumor growth in neuroblastoma cells of A/J mice, which received PolyE-pretreated MDSCs. These findings possibly show that MDSCs fail to stimulate $CD4^+$ T_{REG} cells when they have been exposed to PolyE. PolyE could be potentially beneficial in cancer patients by antagonizing cells that interfere with antitumor immune responses elicited by immunotherapy [604, 605].

Other investigators suggest the role of immunoregulatory cytokine IL-12 in DNA repairs and induction of cytotoxic T-cells in the tumor microenvironment in skin cancer models [606]. In fact, EGCG inhibits UVB-induced immunosuppression and induces repair in mice through stimulation of IL-12. Mechanisms of green tea for chemoprevention in lung cancer include antioxidant activity, phase II enzyme induction, and inhibition of TNF-α expression. EGCG also inhibits UVB-induced infiltration of leukocytes and APC depletion [603, 606, 607]. In addition, topical application of EGCG has been shown to inhibit UVB-induced angiogenesis while inducing cytotoxic T-lymphocytes ($CD8^+$ T-cells) in skin tumors on SKH-1 mice [608].

Synergistic Effect of EGCG Combined with Other Bioactive Compounds and Chemotherapeutics

Recent studies have found synergistic antitumor effect of EGCG in combination with other dietary bioactive compounds like ascorbic acid, curcumin, 6-gingerol, N-acetylcysteine, panaxadiol, pterostilbene, quercetin, sulforaphane, vitexin-2-o-xyloside, raphasatin, EPA-FFA, and proanthocyanidins. Combination of EGCG with these small molecules can synergistically inhibit cancer growth through enhanced bioavailability of EGCG.

Several studies reported that EGCG could sensitize cancer cells to X-irradiation and ionizing radiation in different cell lines like glioblastomas and promyelocytic leukemia HL-60 cells. In addition, EGCG can also improve the chemotherapeutic effect of various drugs such as paclitaxel, capecitabine, cisplatin, docetaxel, and doxorubicin (DOX). Therefore, considering EGCG as an adjuvant therapy can be a practical and efficient approach for cancer treatment [559].

Ginseng

Ginseng (the root of *Panax ginseng*) is a well-known herbal medicine for the treatment of various disorders. The main active components of

ginseng include a series of tetracyclic triterpenoid saponins (ginsenosides), polyacetylenes, polyphenolic compounds, and acidic polysaccharides [609]. Until now, 38 ginsenosides have been purified from ginseng roots, with seven major ones, namely, Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd, comprising more than 80% of the total available ginseng [610]. Ginsenosides can be classified into three groups: the protopanaxadiol group (e.g., Rb1, Rb2, Rb3, Rc, and Rd), the protopanaxatriol group (e.g., Re, Rf, Rg1, and Rg2), and the oleanane group (e.g., Ro) [611, 612]. The acidic polysaccharides are found to be more biologically active. Preliminary studies showed that the neutral polysaccharides contain antitumor activity [613]. A case-control study in Korean population reported that long-term ginseng intake was associated with a decreased risk of different types of cancers [614]. The main active components of red ginseng for cancer prevention are ginsenosides Rg3, Rh2, Rg5, and PPD, which work synergistically [615, 616].

Acidic polysaccharides of ginseng (ginsan) isolated from the ethanol-insoluble fraction of the *P. ginseng* root have also demonstrated anticancer immune modulatory function [617, 618]. Treatment with ginsan (acidic polysaccharide fraction of ginseng) makes splenocytes isolated from unprimed normal mice to be converted into activated killer (AK) cells, which can induce cytotoxic activity on numerous tumor cells comprising NK-resistant murine mastocytoma cell line P815 and NK-sensitive murine lymphoma cell line YAC-1 [619, 620]. Ginsan can be combined with other immunotherapeutics like IL-2 to enhance antitumor effect. Ginsan can stimulate the production of cytokines IFN-γ, IL-2, IL-1, TNF-α, IL-12, GMCSF, and IL-4 to modify the function of AK cells. Macrophages are also necessary as accessory cells for the production of AK cells by ginsan [619]. The immune phenotype of these cells was described to be $Thy1^+$ (thymocyte and peripheral T-cell marker), $AsGM^+$ (NK cell and basophil marker), $CD4^+$, and $CD8^+$ [619].

Ginsan is able to convert macrophages into an M1 tumor inhibitory phenotype [617] as reported in peritoneal macrophages on murine B16 melanoma and fibroblast L929 cells. Generation of NO and ROS by macrophages is modified by inflammatory cytokines; and ginsan-treated peritoneal macrophages significantly enhance secretion of IFN-γ, TNF-α, IL-1β, and IL-6 [617].

Red ginseng acidic polysaccharide (RGAP) increased cytokine secretion by macrophages but did not stimulate their tumoricidal activity on its own [618]. RGAP combined with recombinant IFN-γ possesses an increased synergistic effect on the cytokine production and phagocytic and cytotoxic capacity of macrophages against murine B16 melanoma cells. Activation of the NF-κB pathway has been postulated to be responsible for this synergistic effect [618].

The red ginseng ginsenoside Rg3 also showed stimulatory effects on macrophages and increased the phagocytic index of peripheral blood macrophages resulting in an improved antitumor effect in a mice model of lung carcinoma [621]. Korean red ginseng (KRG) possesses no effect on the accumulation of MDSCs. However, it might inhibit suppressive function of these cells leading to immune activation mediated by T-cell proliferation and cytokines IFN-γ and IL-2 [622]. Altogether, it must be mentioned that the bioactive constituents of *ginseng* demonstrated favorable anticancer immunotherapeutic effects, which are mainly modified via production of tumoricidal macrophages and AK cells.

Anti-inflammatory Effects

Several ginsenosides have been shown to affect inflammatory signaling pathways, thereby inhibiting cancer development [623]. In a chemically induced mouse model of skin carcinogenesis, topical administration of ginsenoside Rg3 suppressed TPA-induced activation of NF-κB and AP-1 and COX-2 expression, accounting for its antitumor effects [624]. 20(S)-Rg3 can inhibit the production of ROS, but not that of NO, and decrease the production of cytokines, such as TNF-α, IL-1β, IL-6, and PGE-2 in LPS-stimulated Raw 264.7 murine macrophages and human keratinocyte (HaCaT) cells [625]. In MCF-7 cells, ginsenoside Rg1 inhibited MMP-9 activity through NF-κB-mediated suppression of breast cell migration and invasion [626].

Ginsenoside Rg5 is also able to suppress NF-κB activity in a lung inflammation model. Rg5 reduced the expression of COX-2, iNOS, IL-1β, and TNF-α in LPS-stimulated alveolar macrophages through inhibition of IL-1 receptor-associated kinases (IRAKs) and IκB kinase-β (IKKβ), subsequently blocking the phosphorylation and nuclear accumulation of NF-κB [627]. Inhibition of NF-κB and subsequent reduction in IL-8 and PGE-2 also have been demonstrated in human embryonic kidney (HEK)-293 cells and HaCaT keratinocytes [628].

Treatment of human esophageal carcinoma cells with ginsenoside Rg3 reduced expression of VEGF, which was associated with the reduced expression of HIF-1α and COX-2 and diminished NF-κB activity [629]. Rg3 combined with gemcitabine significantly reduced the growth rate of Lewis lung carcinoma cells transplanted in C57BL/6 mice by reducing the expression of VEGF [630].

P. ginseng can inhibit chemically induced aberrant crypt foci in mice maybe through anti-inflammatory activities like inhibition of COX-2. Ginseng can also inhibit MMPs and kinase pathways. In addition, it was demonstrated that ginseng activates PPAR-γ and TGF-β1, which are capable to interfere with the inflammation-to-cancer process. The following anti-inflammatory effects of ginsenosides have been reported in cancer models: inhibition of COX-2 and NF-κB in gastric cancer; inhibition of MAPK, NF-κB, and AP-1 in liver, lung, and breast cancer; and inhibition of iNOS, COX-2, and NF-κB in mammary and liver cancer [631].

Compound K (CK) significantly inhibited the secretion and protein expression of MMP-9. The inhibitory effect of compound K on MMP-9 expression was correlated with decreased MMP-9 mRNA levels and reduced MMP-9 promoter activity [632].

Red ginseng inhibited tumor growth by influencing neovascularization and angiogenesis. The angiosuppressive effect of Rg3 could be due to the differential regulation of MMP-2 and MMP-9 activities [633]. Dose-dependent downregulation of MMP-2 and MMP-9 production by Rg3 is thought to be responsible for the inhibition of endothelial cell invasiveness and angiogenesis [633]. Rg3 effectively abrogated the VEGF-dependent neovessel formation, leading to delayed tumor angiogenesis [634]. In a model for gastritis and gastric cancer, treatment of endothelial cells with KRG significantly reduced the expression of inflammatory mediators, including iNOS, COX-2, IL-8, and IL-1β, and angiogenic factors including IL-6, VEGF, platelet-derived growth factor, and MMPs [618].

Role of microRNA in Inflammation-Related Angiogenesis

Recent researches have highlighted a role for microRNAs (miRNAs) – noncoding short RNA molecules (18 to 23 nucleotides) – in controlling gene expression by directing mRNA degradation or repressing post-transcriptional translation, thereby silencing gene expression [635].

A recent study showed that ginsenoside Rh2 caused upregulation of 44 miRNAs and downregulation of 24 miRNAs in human non-small cell lung cancer cells. Interestingly, affected miRNAs were mostly involved in angiogenesis, inflammation, and cell proliferation [636]. Furthermore, Rh2 suppressed miR-21, miR-27b, and miR-31, all of which exhibit pro-angiogenic effects consistent with the reported anti-angiogenic activities of Rh2 [637]. Ginsenoside Rg3 has been shown to regulate VEGF-induced angiogenic response via miRNA modulation [635]. Red ginseng caused a synergistic effect with drug 5-FU for antiproliferative impact on a human CRC model [638]. Red ginseng significantly potentiated the anticancer activities of epirubicin and paclitaxel; thus, their dose and adverse events could be decreased [639]. Rg3 has been demonstrated to block NF-κB signaling and improve the vulnerability of prostate cancer cells to docetaxel and other chemotherapeutics. Also, protective effect of red ginseng in anticancer drug-induced toxicity was reported to be mediated via the regulation of NF-κB activities [640].

Carotenoids

β-Carotene is the main carotenoid isolated from orange and yellow fruits and vegetables.

Lycopene is the main carotenoid found in red fruits and vegetables. The correlation between the high dietary consumption of carotenoids and low risk of prostate cancer has been frequently investigated. The inhibitory effect of β-carotene on the proliferation of human cancer cell lines (PC-3, DU 145, and LNCaP) has been previously demonstrated [641]. Carotenoids have been investigated for their immune-enhancing effects mostly via induction of NK cell activities and increasing leukocyte cell number, CD4/CD8 ratio, and MHC I expression [642]. The antitumor effect of dietary lutein has been investigated in a mammary tumor-bearing mice model. Lutein showed a stimulatory effect on IFN-γ expression while suppressing the expression of IL-10 in splenocytes [643, 644].

Lycopene is a potent antioxidant that can be used as a protective anticancer agent [645]. The antiproliferative and apoptotic effects of lycopene have been shown in prostate cancer cell line (LNCaP) [646], colon cells (HT-29 and T84), and breast cancer cell lines [647]. Both lycopene and β-carotene have been shown to inhibit metastasis in experimental settings, for example, lung metastasis in B16F-10 melanoma cells in C57BL/6 mice and in human hepatoma SK-Hep1-1 cells. Lycopene also decreases the level of VEGF and MMP [648].

In vitro administration of lycopene effectively reduced inflammatory signaling. Lycopene was able to inhibit the mRNA and protein expression of the pro-inflammatory cytokine IL-8 via inactivation of the NF-κB transcription factor through inhibition of the phosphorylation of IKK and IKB and by decreasing the translocation of the NF-Bp65 subunit from the cytosol to the nucleus. Lycopene also decreased the production of TNF, COX-2, iNOS, and IL-6 [649, 650]. The effects of lycopene were correlated with reduced phosphorylation of COX-2, PGE-2, and ERK1/ERK2 [651]. Lycopene also decreased MMP-7 expression in colon cancer cells. The decrease of MMP-7 expression by lycopene was associated with diminished stability and increased E-cadherin expression, showing that MMP-7 may hydrolyze this adhesion molecule. Furthermore, lycopene decreased MMP-7 and c-myc expression by blocking AKT, GSK3, and ERK1/ERK2 phosphorylation [652].

β-Cryptoxanthin was shown to reduce the gene expression of IL-1α in mouse macrophage RAW 264 cells [653]. Both astaxanthin and canthaxanthin exhibited inhibitory activity in relation to cancer development in the urinary bladder, tongue, and colorectum through downregulation of cell proliferation. Another study demonstrated the anti-inflammatory and antitumor effects of astaxanthin in inflamed colon due to modification of the expression of inflammatory cytokines that are involved in inflammation-associated carcinogenesis [654]. Indeed, astaxanthin may aid COX-2 suppression [655]. Other studies reported that in 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis, daily administration of astaxanthin significantly blocked colon carcinogenesis by modifying the expression of NF-κB, COX-2, MMP-2, MMP-9, ERK2, and protein kinase B (Akt) [656]. Lycopene decreased the invasive ability of hepatoma cells by downregulating the activity of NF-κB [657], maybe through suppression of IGF-1 receptor. Lycopene could be efficient in treatment of benign prostate hyperplasia (BPH) via inhibition of NF-κB. On the contrary of the inhibitory effect of lycopene on NF-κB activity, β-carotene stimulated NF-κB in human leukemic (HL-60) and colon adenocarcinoma (LS-174 and WiDr) cells [658]. Astaxanthin attenuated the production of inflammatory markers and cytokines by LPS in vitro (LPS-treated RAW 264.7 cells and primary macrophages) and in vivo (LPS-treated mice) through NF-κB inhibition. Furthermore, astaxanthin thoroughly inhibited all the main signaling molecules involved in NF-κB activation, like IkB kinase phosphorylating activity, IkBa degradation, and the nuclear translocation of the NF-κB p65 subunit [659]. Anti-angiogenic effect of β-carotene was investigated by an in vivo model of B16F-10 melanoma in mice and by in vitro studies [660]. β-Carotene treatment significantly decreased the number of tumor-directed vessels concurrent with reduction of serum VEGF and pro-inflammatory cytokines, e.g., IL-1β, TNF-α, and IL-6. In addition, similar decrease of these cytokines was detected after β-carotene treatment in melanoma cells and found to result from inhibition of c-Rel subunit of NF-κB and AP-1. AP-1 transcription system has been shown to be

blocked by lycopene in MCF-7 mammary cancer cells [661]. The AP-1 complex comprises proteins from the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families, which are connected as homo- (Jun/Jun) or heterodimers (Jun/Fos). It is probable that lycopene and retinoic acid decrease growth factor-induced induction of AP-1 transcriptional activity by changing the composition of AP-1 complexes that bind to DNA [662, 663]. There was a three- to fourfold increase in the expression of c-Jun and c-Fos genes in the lungs of ferrets, supplemented with high-dose β-carotene and exposed to tobacco smoke. This study suggested a possible explanation for the enhancing effect of β-carotene on lung carcinogenesis in smokers, as demonstrated in large intervention studies [664].

Under basal conditions, Nrf1 and Nrf2 are present in the cytoplasm bound to the inhibitory protein Keap1. After challenge with stimulating agents, they are released from Keap1 and translocated to the nucleus [665, 666]. Studies have shown that dietary antioxidants (terpenoids [667]), flavonoids (epigallocatechin gallate (EGCG) [668, 669]), and isothiocyanates may function as anticancer agents by activating this transcription system. However, hydrophobic carotenoids such as lycopene lack any electrophilic group and, therefore, are unable to interact with Keap1. Thus, it seems that oxidative products actively play a role in the induction of the EpRE/ARE (electrophile/antioxidant response element) transcription system by carotenoids. It has been demonstrated that oxidized derivatives, isolated by ethanol from partially oxidized lycopene, transactivated EpRE/ARE in HepG2 human hepatocellular carcinoma cells [670] with a strength resembling to that observed with unextracted lycopene mixture. In contrast, the intact carotenoid exhibited a small insignificant effect [671].

Isoflavones

Isoflavones, such as daidzein and genistein, are mostly found in soybeans. Previous experimental and epidemiological studies suggest cancer protective effects of isoflavones and their metabolites. Genistein was described to downregulate direct cellular cytotoxicity and ADCC. Genistein is able to inhibit tyrosine kinase activity, which is crucially involved in NK cell activation in epidermoid carcinoma A431 cells [672]. Both genistein and daidzein are extensively metabolized in humans and found as conjugated metabolites, mainly glucuronides [673, 674]. Genistein and daidzein glucuronides could increase NK cell-mediated cytotoxicity in human PBMCs at nutritionally achievable concentrations, which were tenfold lower than concentrations of isoflavones used to inhibit tumor cell (MCF-7 and MDA-468 human breast cancer cells) growth in vitro [673, 675]. At higher concentrations, however, genistein decreased NK cell-mediated killing of K562 target cells. In the presence of IL-2, genistein increased NK cell activation at even lower concentrations. The existence of IL-2 may be essential for genistein to increase NK cell activity, and this may be correct for other flavonoids too. Factors determining the effects of genistein on NK cell activity in mice include the duration of exposure, sex, and even generation. The described effects may be of nutritional relevance as isoflavone concentrations after soy consumption are within the range (<2 μmol/L) for which NK cell activation is anticipated. The glucuronides were generally less potent than genistein and daidzein [673]. Genistein administration by oral gavage for 1–4 weeks increased NK cell-mediated cytolysis and cytotoxic T-cell activity in B6C3F1 mice [676].

Moreover, increased host resistance was shown in adult B6C3F1 mice (intravenous injection of B16F10 melanoma cells) treated with genistein, as reflected in lung tumor weight and NK cell modulatory effects [676]. Increased intake of dietary soy has been reported to reduce the severity of macroscopic lung metastasis [677]. In a study in bladder cancer, isoflavone-rich soy phytochemical concentrate (SPC) was shown to have greater anti-metastatic effect in comparison with genistein. Particularly, SPC but not genistein significantly blocked lung metastases through suppression of NF-κB expression in tumor tissues and reduction of circulating IGF-1 levels [678]. Besides decreasing the metastasis of breast cancer cell to lung [679], genistein has been shown to be a useful chemotherapeutic agent to inhibit the development and metastasis

of sex gland cancers such as prostate cancer [680]. Inhibition of MMP-9 by genistein also has been suggested as a possible mechanism for prevention of prostate cancer to bone metastasis [681, 682]. Other genes targeted by genistein in primary stages of breast cancer include MMP-2, MMP-7, and CXCL12, which support invasion and metastasis [683, 684]. Genistein also inhibited the activation of focal adhesion kinase [685] and HSP27 pathway [685], which regulate cancer cell detachment and invasion, respectively. Genistein has been reported for its cytotoxic effect in prostate cancer cell lines LNCaP and PC3 [686], hepatoma cancer cell lines (HepG2, Hep3B) [687], and A431 and Colo205 xenograft tumors, [688, 689]. Genistein can be used combined with conventional therapy such as 5-FU, all-trans retinoic acid (ATRA), and trichostatin A to improve their cytotoxicity and apoptotic activity in human pancreatic cancer cell line (MIA PaCa-2) [690] and human lung cancer cell line (A549) [691, 692]. Genistein at very low concentrations stimulated the proteinase inhibitor 9 (PI-9), which is a granzyme B inhibitor and inhibits the capability of NK cells to lyse breast cancer cells [693] with an opposite activity in high concentrations [676]. Moreover, it seems that polyphenol-stimulated NK cytotoxicity depends on the cell type. Genistein has also shown to decrease in vitro cytotoxic activity of NK cells in melanoma and breast cancer cells [524, 693] and, in contrast, was found to increase NK-mediated cytotoxicity in in vitro and in vivo tumor models [676, 694, 695].

Quercetin

Quercetin is a well-known flavanol, which has been shown to inhibit NK cell killing activity in peripheral blood lymphocytes from human donors. However, high doses of quercetin could cause pro-apoptotic or cytotoxic effects through the inhibition of Ca^{2+} channels and Na+/K+ ATPase activity [122, 696]. More clearly, indirect NK cell stimulation by quercetin resulting in augmented IFN secretion has been reported in low doses of quercetin. Quercetin improved NK cell activity in BALB/c mice treated with WEHI-3 leukemia cells and oral quercetin [697].

Quercetin stimulated NK cell activity through inhibition of protein kinase C (PKC), PI3K, and HSP70 in target cells while increasing the expression of NKG2D ligands [698, 699]. Some chemotherapeutics were reported to increase the expression of NKG2D and HSPs, thereby decreasing cell vulnerability to NK cell-mediated cytolysis. It has been reported that quercetin can induce the expression of NKG2D ligands, MHC class I-related chain B (MICB), UL16-binding protein 1 (ULBP1), and UL16-binding protein 2 (ULBP2) while downregulating the expression of HSP70 in K562 (erythroleukemia), SNU-1 (gastric carcinoma), SNU-C4 (colon cancer), and human Raji (Burkitt's lymphoma) target cells, together reflected in increasing cell susceptibility to NK-92-mediated lysis [698]. It has been suggested that increased NKG2D ligand expression was mostly responsible for the inhibitory effect of quercetin on NF-κB and PI3K [698]. Quercetin demonstrated an antiproliferative effect through the induction of apoptosis by disturbing the MMP system [700, 701]. In addition, quercetin can be administrated combined with other chemotherapeutic agents such as doxorubicin to enhance their cytotoxic effects on liver cancer cells (SMMC7721 and QGY7701) as well as to provide protection for non-tumoral liver cells from toxic effects of free radicals [702].

Quercetin is able to reduce the number and size of polyp in the Apc (Min/+) mouse through reduction in macrophage infiltration [703]. In addition, treatment with quercetin prior to intraperitoneal injection of EAT tumor cells stimulated macrophage spreading, suggesting that this compound affects the tumoricidal activity of macrophages [704]. In vivo, tumor-bearing mice treated with quercetin showed an improvement in the phagocytic activity of peritoneal macrophages [697].

β-Glucan

β-Glucan is a polymer made of D-glucose molecules that are connected by linear β-glycosidic bond with side branches that are different based

on their sources [705]. β-Glucans, including zymosan, laminarin, lentinan, and pleuran, are found in mushroom, barley, cereals, and seaweeds as well as bacterial and fungal cell wall. The anticancer effect of β-glucan is chiefly because of its immunomodulatory effect rather than its direct cytotoxic activity. A range of β-glucans have been described as immunomodulators [706]. β-Glucans are able to induce the immune system effector cells, mostly macrophages, monocytes, neutrophils, NK cells, and DCs via their interaction with glucan-specific receptors, such as dectin-1, TLR, and CR3 (complement receptor 3 or CD11b/CD18), expressed by these cells [707]. In addition, they can increase the phagocytic effects of neutrophils, NK cells, and cytotoxic T-lymphocytes. β-D-Glucans have been demonstrated to stimulate the secretion of pro-inflammatory cytokines (IL-1α/IL-1β, TNF-α, IL-2, IFN-γ, and IL-12) that stimulate antitumor immune response as well as NO and H_2O_2 by activated macrophages that demonstrated antitumor effect [708]. The effect of natural β-glucan, schizophyllan, combined with chemotherapy was investigated on the survival rate of patients with ovarian cancer [709]. Furthermore, Maitake D-fraction found in *Grifola frondosa* (Maitake mushroom) has been reported to decrease the size of tumors, primarily in the lung, liver, and breast, in more than 60% of treated patients [710]. Moreover, supplementation with 5.4 grams *Ganoderma* polysaccharides per day for 12 weeks boosted immune responses in patients with lung and colorectal cancer [711, 712]. β-Glucans combined with mAbs RMA-S-MUC1 subcutaneously implanted in C57Bl/6 mice improved complement receptor 3 (CR3)-mediated phagocytosis of ic3b (inactivated C3b)-opsonized tumor cells by effector granulocytes and enhanced tumor recession in treated animals [713]. Lentinan, derived from *Lentinus edodes*, was shown to induce apoptosis in hepatoma H22-bearing mice [714], cervical carcinoma HeLa cells, and hepatocellular carcinoma (HepG2 and SMMC-7721 cell). Furthermore, lentinan induced antitumor immune responses through enrollment of immune cells, mostly macrophages and T-lymphocytes, into TME to attack tumor cells and release inflammatory chemokines (TNF-α, IL-2, IL-1β, TGF-β, IP-10, M-CSF, and TREM-1). The immunomodulating effects of arabinogalactan (AG) and fucoidan (FU) in vitro have been investigated in mouse spleen lymphocytes, which turned cytotoxic after treatment with AG and FU. Novel maloyl glucans have been isolated from aloe vera gel (*Aloe barbadensis*) – veracylglucan B possesses both anti-inflammatory and antiproliferative effects, while veracylglucan C has merely shown anti-inflammatory effects and appears to complement the actions of veracylglucan B [715].

Withania somnifera

Withania somnifera (WS), also known as Ashwagandha, has been a part of Ayurvedic medicine for many centuries. WS has been reported to be efficient in arthritis, cancer, and mental disorders [716, 717]. Steroidal lactones, including withanolides and withaferins, are the most biologically active components [716]. Among them, withaferin A (WA) and withanolide A have been investigated for anticancer and immunomodulatory effects, respectively [718, 719].

Along with its antitumor effect, treatment of tumor-bearing mice with withanolide A led to the polarization of T_H1 cells and subsequent increase in the production of pro-inflammatory cytokines (IFN-γ and IL-2) while reducing the polarization of T_H2 cells [720]. Moreover, there was a significant increase in the proliferative activity of $CD4^+$ and $CD8^+$ T-cells present in the serum of WS-treated mice. In response to stimulation with concanavalin A (Con A) and LPS, proliferation of T-cells and B-cells was also significantly increased with WS treatment. Treatment with WA not only increased NK cell population in one study but also increased its cytotoxic activity. In addition, APCs purified from blood samples of tumor-bearing mice showed an enhanced maturation and expression of co-stimulation markers (CD80, CD40, and CD40L) on T-cells [720], suggesting the effective role of WS in DC-mediated activation of T-cells – all of which may be involved in antitumor function of WS. WA treatment induced tumor rejection

and protection from rechallenge. This indicates that WA can build immunological memory in Ehrlich ascites carcinoma model. A possible mechanism of tumor rejection could be attributed to macrophages because WA increased the frequency of peritoneal macrophages, and transfer of these macrophages from cured mice caused tumor rejection. In a breast cancer model, WA induced immunogenic cell death (ICD) in cancer cells through expression of HSPs such as HSP70, HSP90, and calreticulin on the membrane of tumoral cells. All of these ICD mediators bind to receptors on DCs, leading to activation and maturation of DCs and the production of inflammatory cytokine IL-12 [721]. Of note, WA could diminish the function of the tumor inhibitory immune cell type, i.e., myeloid-derived suppressor cells (MDSCs), to generate ROS known to mediate the suppressive effect of MDSCs on T-cells [722].

Flavone Acetic Acid (a Synthetic Flavonoid)

Synthetic flavone acetic acid (FAA) has been frequently investigated for its antitumor activities. In particular, it has the ability to induce NK cell activity [723]. FAA increased NK cell-mediated killing activity in both healthy and tumor-bearing mice [723] as well as cancer patients [724]. It has been postulated that an indirect mechanism (e.g., induction of cytokines), rather than a direct interaction of FAA with NK cells [725], is responsible for the discovered effect. In mouse renal cancer, intravenous or intraperitoneal administration of FAA increased NK cell activity in the spleen, liver, lungs, and peritoneum and was synergistically enhanced by co-administration of IL-2 [725]. The first report on the enhancing effect of FAA on NK cell function in humans came from a study with six cancer patients undergoing a weekly treatment with FAA. Three out of the six patients showed a considerably enhanced NK cell activity after treatment [724]. In another trial, NK cell activity not only remained unchanged after treatment with FAA in cancer patients but even significantly reduced 24 h after treatment [726]. The synergistic activity of FAA and IL-2 [725] was subsequently studied in 26 melanoma patients. In 23 of 26 patients, NK activity was significantly enhanced (2–20-fold higher cytotoxicity) during combined treatment with FAA and IL-2. However, large variations in NK cell activity were observed in patients over the duration of the trial [727]. Of nine cancer patients receiving 1–6 courses of FAA infusions, enhanced NK cell activity was reported in only three patients, while six others were unresponsive to treatment [728].

However, intravenous FAA in the abovementioned trials differed completely from the oral intake of flavonoids through diet or supplements. After intravenous injection, compounds are 100% bioavailable, which surpass the usual maximum plasma concentrations of dietary flavonoids. A possible mechanism of action by which FAA induces NK cell activity is through induction of cytokines, including IFN-α, thereby improving NK cell function.

Phenoxodiol (a Synthetic Flavonoid)

Phenoxodiol is a synthetic analog of genistein [729]. Phenoxodiol could induce NK cell function and their perforin content in human PBMCs from healthy donors, thereby increasing cytotoxicity of NK-sensitive K562 cells. The increased cytotoxicity of phenoxodiol-treated cells was more prominent in PBMCs from cancer patients than in those from healthy volunteers. On the contrary, genistein and daidzein only marginally stimulated PBMC cytotoxicity [675]. In a previous experimental in vivo study, the effects of phenoxodiol genistein and daidzein were investigated in tumor-bearing mice. Only phenoxodiol and only at high-dose of 20 mg/kg body weight was able to enhance the cytolytic activity of splenocytes against NK-sensitive target cells (CT-26 and YAC-1) [675].

Polymethoxylated Flavones

Treatment with a mixture of polymethoxylated flavones derived from orange peel oil in high doses mildly downregulated NK cell activity

with no effect on humoral immunity [730]. These findings suggest that consumption of high-dose citrus fruit during certain conditions like tamoxifen therapy of mammary tumors must be avoided. Polymethoxylated flavones, such as nobiletin, tangeretin, and sinensetin, from the peel of citrus fruits, have been reported to potentiate the cytotoxicity of KHYG-1 (NK leukemia cells that exhibit high cytolytic activity against K562 target cells [731]) by enhancing the expression of granzyme B [731]. Among them, nobiletin was also able to increase the levels of IFN-γ, perforin, granzyme A, and granzyme B in KHYG-1 cells [731]. The important role of granzyme B in nobiletin-mediated cytolysis has been confirmed in that study. It must be noted that nobiletin increased phosphorylation of cAMP response element-binding protein (CREB) while controlling the phosphorylation of ERK1/ERK2 and p38 MAPK [731].

Apigenin and Amentoflavone

Apigenin is found in common fruits and vegetables, such as parsley, onions, oranges, tea, chamomile, wheat sprouts, apple, guava, tomato, and broccoli, and in some seasonings. Studies have reported its antitumor effects. Topical application of apigenin prior to UV irradiation prevents UV-induced tumorigenesis in mice. In addition, it exhibited antiproliferative effects on breast cancer cell lines that expressed different levels of HER2/neu. It induced apoptosis in HER2/neu-overexpressing breast cancer cells. Apigenin has been shown to inhibit cancer cell proliferation and transcriptional activation of VEGF in A549 lung cancer cells [732–737]. Amentoflavone is a biflavonoid formed out of two apigenin units [738]. It is present in *Ginkgo biloba*, Saint John's wort [739], and *Nandina domestica* [740]. Treatment with amentoflavone increased NK cell activity in splenocytes in control and tumor-bearing BALB/c mice [741]. Tumor-bearing controls showed weaker and delayed NK cell activity in comparison with amentoflavone-treated mice [741]. NK cell activity was investigated in splenocytes isolated from tumor-bearing mice incubated with K562 target cells. Furthermore, antibody-dependent cellular cytotoxicity (ADCC) was significantly improved in amentoflavone-treated mice [741]. Taken together, amentoflavone effectively increased lymphoid cell proliferation and effector cell functions by inducing the production of IL-2 and IFN-γ in tumor-bearing mice [741].

Proanthocyanidins

Proanthocyanidins derived from grape seeds have different strong immunomodulatory properties. Ultraviolet B (UVB), as a part of UV irradiation, causes immunosuppression which can be inhibited by proanthocyanidins through the induction of IL-12 in mice [742]. In addition, proanthocyanidins can inhibit UVB-induced immunosuppression by inducing $CD8^+$ effector T-cells and reducing regulatory $CD4^+$ T-cells. Proanthocyanidins make UVB-exposed mice to secrete higher levels (five- to eightfold) of T_H1 cytokines from $CD8^+$ T-cells and lower levels (80–100%) of T_H2 cytokines from $CD4^+$ T-cells [743]. Of note, proanthocyanidins increase the frequency of $CD4^+CD25^+FoxP3^+$ regulatory T-cells while decreasing the frequency of $CD4^+IL\text{-}17^+$ pathogenic T-cells. Downregulation of IL-17 secretion and enhancement of Foxp3 expression because of proanthocyanidin treatment have been reported in vivo.

Organosulfur Compounds

Garlic is a rich source of organosulfur compounds (OSCs), including allicin, diallyl sulfide, and diallyl disulfide, which contain, respectively, mono-, di-, and polysulfide functional groups [744]. Garlic and its compounds are capable to facilitate stimulation of immune effector cells to promote antitumor immunity [745]. Aged garlic extract (AGE) has been reported to stimulate phagocytosis by macrophages and cytotoxic activities of T-lymphocytes [746] in sarcoma-180-bearing mice. In addition, it can increase the secretion of pro-inflammatory cytokines (IL-2,

IL-12, TNF-α, and IFN-γ) and the frequency of NK cells. However, diallyl disulfide, diallyl sulfide, and allyl methyl sulfide exhibited an inhibitory effect on the release of TNF-α, IL-10, and NO generation in LPS-stimulated RAW 264.7 macrophages [747]. Some dietary phytochemicals like sulforaphane are powerful stimulators of phase II/detoxifying genes, and this effect is dependent on nuclear factor erythroid 2-related factor 2 (Nrf2) [748]. In fact, sulforaphane is able to stabilize Nrf2 [749].

Capsaicin

Capsaicin is the dominant pungent component present in red chili pepper [750, 751]. The antiproliferative effects of capsaicin through several mechanisms including production of ROS and disruption of mitochondrial membrane and release of cytochrome c have been reported in some cancer cell lines, such as leukemic cells (NB4 and Kasumi-1 cells) [752], prostate cancer cell line PC-3 [753], and human colon adenocarcinoma Colo205 cells [754]. The anti-angiogenic effects of capsaicin have been shown via its suppressive effects on VEGF. Capsaicin is able to inhibit NF-κB and STAT3 transcriptional pathway that play a vital role in inflammation and tumor growth [755, 756].

Bromelain

Bromelain is a mixture of proteolytic enzymes purified from pineapple (*Ananas comosus*). It has been approved as an anti-inflammatory agent for post-surgical conditions and infection. Immunomodulatory effects of bromelain include (1) induction of CD2-mediated T-cell activation [757], (2) increasing T-lymphocyte proliferation in splenocytes without significant effect on purified $CD4^+$ and $CD8^+$ T-cells [758], and (3) decreasing the production of pro-inflammatory cytokines, such as IL-2, IL-6, IL-4, IFN-γ, and G-CSF, from inflamed tissues [759]. The immunostimulatory effect of bromelain was only demonstrated on the healthy immune system when combating foreign antigens [760, 761]. Also, bromelain is able to stimulate the oxidative explosion in neutrophils by increasing intracellular ROS that induce DNA destruction, thereby enhancing the cytotoxic effect of neutrophils on tumor cells [762]. The antitumor and cytotoxic effect of bromelain has been shown in mouse skin papilloma through inhibition of NF-κB and COX-2 expression [763]. Its cytotoxic effect has been shown on melanoma B16F10-Nex2 cells [764] and human cholangiocarcinoma cell lines (TFK-1, SZ-1) as well [765]. Bromelain has the ability to decrease the expression of CD44 surface marker, which is involved in tumor proliferation [766]. Of note, bromelain treatment led to a significant reduction in invasion, migration, and adhesion of glioma cells without any adverse effect on marginal cells [767].

Betulinic Acid

Betulinic acid (Bet A) is a naturally occurring triterpenoid present in several plant species such as the white birch (*Betula pubescens*). Bet A has been investigated for its cytotoxic effects on melanoma cells [768], neuroblastoma tumor cells [769], glioma cells [770], human leukemia HL-60 cells [771], malignant head and neck squamous cell carcinoma SCC25 and SCC9 cell lines [772], and colon cancer cells [773]. Of note, Bet A is able to inhibit the secretion of IL-6, COX-2, and PGE-2 in LPS-induced PBMCs via downregulation of NF-κB signaling [774, 775].

Zerumbone

Zerumbone is a sesquiterpene in the rhizomes of shampoo ginger. Zerumbone has immunomodulatory activity via modulation of MAPK and NF-κB pathways [776] and cytokine secretion [777]. It has been demonstrated to downregulate production of different inflammatory mediators, mainly NO, COX-2, PGE-2, and iNOS in macrophages [778]. Moreover, this potent immunomodulator has been investigated for its anticancer effects and suggested to be helpful in cancers of the breast, bone marrow, liver, lung, cervix, colon, prostate, pancreas, and skin [778–784].

Noni Fruit

Morinda citrifolia (noni) is a Hawaiian plant used for cancer. Its polysaccharide-rich substance has been shown to possess antitumor effect in the Lewis lung tumor model, resulting in improvement of the host immune system through affecting the production of cytokines (TNF-α and IFN-γ) and nitric oxide. Two glycosides, 6-*O*-(β-d-glucopyranosyl)-1-*O*-octanosyl-β-d-glucopyranose and asperulosidic acid, were purified as active compounds from noni juice. Both compounds were efficient in downregulating TPA- or EGF-induced cell transformation and associated AP-1 activity [785].

Flavanols

Other flavanols like myricetin have been investigated in the context of antitumor immunology. Myricetin potentiated the ability of NK-92 cells to lyse K562 erythroleukemia target cells [786].

Naringenin

Naringenin is the major flavanone in grapefruit. It was reported to increase the expression of NKG2D ligands in human Raji (Burkitt's lymphoma) cells [787]. MICA, MICB, ULBP1, and ULBP2 protein expressions were also increased compared with untreated control cells [787]. Although quercetin exhibited weaker but similar effect on NKG2D ligand expression, luteolin (flavone), kaempferol (flavonol), taxifolin (flavanonol), apigenin (flavone), and hesperetin (flavanone) did not show modulation of NKG2D ligand expression [787].

Chrysin

Chrysin is the main flavanone of *Passiflora incarnata* (also known as passion flower) [788]. It can be found in natural products like propolis and honey [789]. Chrysin has been reported to have anti-inflammatory, antioxidative, and chemopreventive activities [789]. Oral administration of chrysin in a murine leukemia mouse model increased populations of T- and B-lymphocytes and enhanced phagocytosis by macrophages as well as NK cell-mediated cytotoxicity. After chrysin treatment, the viability of WEHI-3 cells (murine leukemia cells) was reduced. Splenocytes isolated from WEHI-3-injected leukemic BALB/c mice after chrysin treatment exhibited an enhanced NK cell toxicity toward YAC-1 target cells [789].

Tangeretin

The flavone tangeretin is found in citrus fruit peel [790]. Tangeretin treatment in female C3H mice reduced lymphocyte counts, suggesting an inhibitory effect of tangeretin on cell proliferation and differentiation of NK cells [791]. Tangeretin also antagonized the tumor-suppressive effects of tamoxifen in MCF-7/MCF-6 tumor-bearing mice by reducing the number of NK cells and NK cell activation through lymphokines [790]. The in vivo antitumor effect of tangeretin has been shown in DMBA (7,12-dimethylbenz(a) anthracene)-induced breast cancer-bearing animals [792]. The antiproliferative and anti-angiogenic effects of tangeretin in A549 human lung cancer cell line have been attributed to downregulation of IL-1β-induced COX-2 expression. Moreover, it has the capability to enhance the levels of non-enzymatic antioxidants (ascorbic acid, vitamin E, and GSH) and reduce the serum levels of tumor markers [793, 794].

Silymarin

Silymarin has shown both antitumoral and cytoprotective effects. It has been reported that silymarin can inhibit NF-κB activation [795]. Another study has shown the biphasic effect of silymarin on Jurkat cells, a human peripheral blood leukemia T-cell line [796]. Low dose of silymarin increased cell proliferation, while high doses caused inhibition of DNA synthesis and significant cell death [797].

Alkaloids

Caffeine is a major phytochemical, which belongs to the alkaloid class. Using the B16F-10 melanoma cell-induced experimental metastasis model, oral and intraperitoneal caffeine administration significantly decreased tumor size [798]. Investigation using a spontaneous transgene-induced mammary tumor model provided further evidence of inhibition of metastasis by caffeine [799].

6-Gingerol

6-Gingerol is the pungent phenolic compound derived from ginger (*Zingiber officinale*). 6-Gingerol demonstrated antiproliferative effect by stimulation of apoptosis against several tumor cell lines such as OSCC and cervical HeLa [800]. Moreover, 6-gingerol showed an anti-metastasis effect on lung B16F10 melanoma in vivo. Inhibition of angiogenesis occurred through downregulation of VEGF. Also, it exhibited its inhibitory effect on COX-2 expression by downregulation of p38 MAPK and NF-κB in vivo [801].

Kaempferitrin

The antitumor and immunostimulatory effects of bioactive flavonoid kaempferitrin from *Justicia spicigera* have been reported in human cervical carcinoma cells (HeLa) [802]. More precisely, kaempferitrin is able to stimulate antitumor immune responses by inducing phagocytic activity of human macrophage in vitro, enhancing the levels of NO and generation of H_2O_2, and stimulating NK activity.

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Nutrition and Cancer

13

Laleh Sharifi

Contents

Key Points

- Cancer is the second leading cause of death globally and is assumed to be a major global health concern.
- There is a multitude of studies about the influence of the different components of foods, diets, breastfeeding, fatness, and physical activity on mutagenic processes.
- Nutrition and cancer have a two-sided and complex association. Nutrition has been reported to increase the effectiveness of the immune system and to prevent cancer development or, conversely, promote malignancy.
- Cancer-protective foods such as whole grains, fruits, non-starchy vegetables, fish, and dairy products contain nutrients and non-nutrient bioactive components that are involved in the mechanisms

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for enhancing immunologic function, inducing apoptosis, enhancing antiproliferative function, donating electron to free radicals, activating carcinogen-metabolizer enzymes, as well as synthesizing retinoic acid.
- Those types of foods, with cancer-developing effects like red meat, processed and fast foods, alcohol, as well as sugar-sweetened beverages, exert carcinogenic effects mostly by causing obesity and overweight, unbalanced hormones, inflammation, oxidative stress, and lipid peroxidation.
- Cancer-preventing recommendations include keeping a healthy weight, having a physically active life, and following a diet rich in foods of plant sources and limited in red meat, fast foods, alcohol, and sugar-sweetened drinks.

Introduction

Cancer is caused by uncontrolled growth of cells which are able to invade other parts of the body [1, 2]. Symptoms of cancer depend on the location of the cancer. However, general symptoms of cancer include unintentional weight loss, excessive fatigue, fever, and skin changes [3].

Cancer is the second leading cause of death globally and is assumed to be a major global health concern. It is estimated that the number of affected people will reach 21.7 million by 2030 around the world [4]. In 2030, the global economic burden of cancer, including medical and nonmedical costs and income losses associated with work absences, is expected to be 458 billion USD [5].

There are many data showing that lifestyle and some nutrients are associated with cancer development as they are connected with other chronic disorders [6]. Epidemiological data from the past decade have demonstrated that a healthy diet can prevent up to 40% of all malignancies [7, 8]. Nevertheless, western lifestyle and its increasing trend in other places lead to decreased physical activity and fatness. In case of continuing current tendency, obesity and overweight have the potential to surpass smoking as the main risk factor for cancer.

The purpose of this chapter is to provide an overview of the effects of nutrition on the progress of cancer and its role as a part of therapeutic regimens.

Cancer Development

The malignant character of cancers results from a failure to preserve the balance between cell proliferation and apoptosis. Cancer mortality is caused by the uncontrolled growth of tumoral cells within body tissues and their subsequent damages [9]. The section below is a brief discussion of the cellular and molecular mechanisms associated with cancer.

Cellular and Molecular Mechanisms Associated with Cancer

Defects in DNA Repair

Deoxyribonucleic acid (DNA) is constantly influenced by environmental factors including ultraviolet (UV) radiation and cigarette toxic components and by harmful intracellular metabolites such as hydrogen peroxide and reactive oxygen species (ROS) that cause damage in DNA integrity and structure. Defects in cell ability to repair DNA damages such as DNA damage checkpoints and telomere repairs result in genomic instability [10]. Genomic instability leads to amplitude unfavorable mutations which can predispose a cell to become malignant [11]. The host physiological reactions to DNA damage are modulated by dietary elements, physical activity, and body fat content, which are discussed in the next sections.

Oncogenes

In a healthy condition, cell proliferation is regulated by a series of genes involved in cell-division cycle, programmed cell death (apoptosis), and survival signals like tissue growth factors and hormones as well as their receptors. Oncogenes are blemished genes that are

nonstandard versions of genes, which are normally responsible for cell proliferation. Oncogenes are the result of a set of mutations or epigenetic changes that progress the malignancy via the unbalanced proliferation of cells. Only a small group of all malignancies is associated with the inheritance of a cancer-linked germ-line mutation (5–10 percent of all cancers). In familial cancer cases, only one mutation contributes to developing cancer. The presence of these mutations does not ultimately lead to cancer but increase the risk of cancer development in relation to the general population. However, inherited mutations are a small proportion of cancer-risk factors, but the effect of other risk factors is the same as other people who have not inherited these mutations. For example, low physical activity increases the risk of breast cancer equally in both people with inherited BRCA1/2 mutation and people with no heritage of BRCA1/2 (7). Hence, identifying the people with these inherited genes is very vital to run preventive programs for the carriers and their family members.

Cancer Hallmarks

Phenotypic features of cancers are known as cancer hallmarks. In spite of the numerous pathways leading to genomic instability and its subsequent cancer development, the hallmarks of all cancers are relatively limited. Hallmarks of cancer include tumor-promoting inflammation, genomic instability and mutation, enabling replicative immortality, resisting cell death, activating invasion and metastasis, inducing angiogenesis, degranulation cellular energy, sustaining proliferative signaling, evading growth suppressors, and avoiding immune destruction. Genomic instability and Inflammation are two main characteristics, which enable other hallmarks to present.

Immune Response to Cancer

Malignant cells arise from body cells (self); however, they can induce immune responses. Indeed, products of oncogenes and mutated genes are dissimilar to usual cellular proteins; therefore, malignant cells elicit immune responses.

The theory of immune surveillance of cancer states that the host immune system detects and eliminates transformed cells before they grow into tumors and kills tumors after they formed.

Histopathologic findings show that tumors are surrounded by infiltration of mononuclear cells including T lymphocytes, natural killer (NK) cells, and macrophages. Furthermore, tumor-activated macrophages and lymphocytes are found in lymph nodes around the site of the tumor. Both innate and adaptive immunities are involved in tumor-fighting strategies.

CD8+ Cytotoxic T Lymphocytes (CTLs)

Peptides from tumor antigens are presented by class I histocompatibility molecules (MHC I). Only within a few minutes, the CTL transports granule proteins into the target cell, causing apoptotic death of the malignant cell.

Natural Killer (NK) Cells

One of the mechanisms where a tumor cell escapes the immune system is the loss of the MHC I to become undetectable by CTLs. Natural killer (NK) cells as innate immune cells destroy various types of tumor cells, mainly the transformed cells expressing lower MHC I on their surface.

Macrophages

Macrophages are another type of innate immune cells which, based on their activation state, play two contradictory roles, i.e., inhibiting and helping the development and spread of cancers. Typically, activated M1 macrophages are able to kill many tumor cells.

Antibodies

The role of antibodies against various tumor antigens has been demonstrated mostly in vitro. Data is lacking for the effectiveness of humoral immune responses against cancers.

Established Causes of Cancer

Factors known to alter the normal cellular processes and substantially lead to cancer include inherited mutations, steroid hormones, insulin and insulin-like growth factors, inflammation, and oxidative stress. Smoking, UV light radiation, alcohol drinking, and infections are among exogenous causes of cancers.

The Role of Nutrition in Cancer Development

A healthy immune system should detect and destroy the malignant cells. Breaking host immune surveillance leads to cancer progression. Cancer is more frequent in immunodeficient patients as their immune system is unable to defend against pathogens and cancer cells.

There is a multitude of studies about the influence of different components of foods, diets, breastfeeding, fatness, and physical activity on mutagenic processes. Nutrition and cancer have a two-sided and complex association. Food and nutrition have been reported to increase the effectiveness of the immune system and prevent cancer development or, conversely, promote malignancy.

Naturally, the immune system, to progress its defense mechanisms against pathogenic microorganisms and tumors, uses cytokines and reactive oxygen and nitrogen species that are toxic to target cells. Long-time production and elevated amounts of these materials cause chronic inflammation. Inflammation may trigger the promotion phase of carcinogenesis and increase the risk of malignancy.

Antioxidants as vitamins A and C, selenium, zinc, probiotics, and n-3 PUFAs as nutrition components enable the immune system to precisely target the infected or transformed cells and not damage the adjacent cells.

Whole Grains

The bran and germ of whole grains contain nutrients such as zinc, selenium, copper, and vitamin E. Furthermore, they have non-nutrient bioactive components including dietary fiber, phytoestrogens, and phenolic acid. Studies have shown anticancer effects of these components [12, 13]. For example, plasma levels of alkylresorcinols (a biomarker for whole-grain intake) are associated with a reduced risk of colorectal cancer [14]. Additionally, whole grains may bind to carcinogen components and reduce the risk of developing cancer.

Different mechanisms to mediate anticancer effects of dietary fiber have been suggested. Bowel microbial fermentation of fibers produces short-chain fatty acids, and their antiproliferative role for colon cancer cells is demonstrated [12, 15, 16]. Insulin resistance is one of the risk factors for colon cancer, and high amounts of fiber can lessen insulin resistance; therefore, fiber use leads to lower risk of colon cancer [17, 16]. Furthermore, fiber reduced the intestinal transit time and increased fecal bulk which helps to lower contact of colon epithelium with carcinogen agents of fecal bulk and subsequently reduced the risk of colon cancer [18].

In spite of cancer-preventing effects of whole grains, they are at risk of contamination with aflatoxin. Metabolites of this mycotoxin are genotoxic to cells and increase the risk of hepatocellular cancer [19–22]. Governments are responsible for controlling the safe storage of food in order to inhibit aflatoxin formation. In addition, people should be aware of aflatoxin hazard and avoid eating moldy grain and legumes.

Non-starchy Vegetables and Fruits

Non-starchy vegetables contain a number of anticancer components including fiber, vitamins, selenium, isothiocyanates, carotenoids, phenols, plant sterols, flavonoids, dithiolthiones, glucosinolates, indoles, limonene, and allium compounds. Many of these components are antioxidants that prevent exposure to ROS; for example, vitamins C and E donate an electron to free radicals in order to block their cellular damage [23–25]. It is probable that a combination of these components is responsible for reduced risk of cancer.

There are a series of studies suggesting molecular mechanisms for anticancer effects of carotenoids including beta-carotene, lycopene, and beta-cryptoxanthin [26–28]. They prevent some oncogenic mechanisms and inhibit cancer-cell development because of their role in the activation of carcinogen-metabolizer enzymes, the synthesis of retinoic acid [29, 30], enhancing immunologic function, inducing apoptosis; their

antiproliferative functions [31–33]; as well as their role as an antioxidant [34, 35].

Epidemiological studies found associations between consumption of vegetables and fruits and lower risk of several cancers involving mouth, pharynx, larynx [36], nasopharynx [37], esophagus [38], bladder [39], colorectum [40], and prostate cancers [41].

Citrus fruit includes multiple anticancer nutrients such as vitamin C, folate, flavonoids, and fiber. Consumption of citrus fruit has been associated with reduced risk of gastric cancer [24, 42].

There are data about the effect of fruits and vegetables on lung carcinoma and their mechanism of action. The results of these studies suggested that an increased uptake of fruits and vegetables is associated with a modest reduction in the risk of lung cancer. The findings were similar in the current smoker, past smoker, and never smoker groups [43]. Also, there are data about the role of carotenoids [44] and vitamin D [45] on lung cancer prevention.

Isoflavones (diadzein and genistein) have a similar structure to 17-beta estradiol and show elevated affinity to the estrogen receptor. Data from clinical trials and observational studies show that the use of exogenous estrogens increases the risk of lung cancers [46]. Estrogen receptors are present in both healthy and malignant lung tissues [47]. Estrogen has an inducing effect on non-small-cell lung cancer (NSCLC) [48].

The effects of vegetables in breast cancer are probably mediated through a hormone-dependent mechanism. Epidermal growth factor receptor (EGFR) is expressed frequently in estrogen-receptor-negative (ER-negative) breast cancer patients, and some vegetable constituents have been suggested to decrease the EGFR expression and reduce the risk of ER-negative breast cancer [49].

Red Meat

Prolonged and high-temperature grilling or barbecuing of meats leads to the production of heterocyclic amines and polycyclic aromatic hydrocarbons which have been shown in experimental studies to associate with a variety of cancers including colorectum [50], nasopharynx [51], lung [52], and pancreatic cancers [53]. Exposure to tobacco smoke is a principal risk factor for lung cancer. Components of tobacco smoke such as nitrosamines and polycyclic aromatic hydrocarbons are found in grilled meats [54, 55].

Using high-temperature cooking for red meats increases the production of glycation end products. Glycation end products appear to increase the risk of cancer [56].

Hem iron has an important role in oxygen transport, oxidative phosphorylation, DNA synthesis, and cell growth. Enhanced consumption of meat and its products in people who are not at risk of iron deficiency is likely to provide higher amounts of bioavailable iron. Higher amounts of iron are associated with the synthesis of ROS, cause DNA and cellular damage, and promote tumorigenesis [57]. Moreover, hem iron has been found to induce carcinogenic N-nitroso compounds [58]. These components are associated with cancer progression in animal models [59, 60].

Fish

Fish includes high concentrations of selenium and vitamin D, which have the potential to decrease the growth of cancer cells [61–63]. Furthermore, fish and mostly fatty fish include high amounts of long-chain omega-3 fatty acids (eicosapentaenoic and docosahexaenoic acids). Long-chain omega-3 fatty acids are associated with slower cancer cell growth by decreasing the production of inflammatory n-6 PUFA-derived eicosanoids [64, 65]. In addition, these fatty acids modulate the estrogen metabolism, signal transduction, and function of transcription factor [66]. It was found in an animal model study that omega-3 fatty acids through reducing inflammation and oxidative stress in the liver exert protective effects against hepatocellular carcinoma [67]. However, carcinogenic components of polycyclic aromatic hydrocarbons and heterocyclic amines can be found in grilled or barbecued fish [68], and there are some experimental data linking these chemicals to gastric cancer [69].

Dairy Products

Consumption of dairy products is inversely associated with the risk of colorectal and breast cancers [70, 71]. The antitumor effect of dairy products has been mostly attributed to their high calcium content. Calcium plays a significant role in cancer prevention through modulation of cell proliferation, differentiation, as well as apoptosis [72–74]. Intracellular concentrations of calcium are essential to cytotoxic T lymphocytes (CTL) and natural killer (NK) cells for killing cancer cells. On the other hand, proliferation and apoptosis of cancer cells are associated with the intracellular calcium content [75]. Also other components of dairy products including butyrate, conjugated linoleic acids, lactoferin, and vitamin D are linked to reduced risk of malignancy [76, 77]. However, there are some disagreements about the role of dietary intake or linoleic acid biomarkers in cancer prevention [78].

High consumption of dairy products is associated with increased risk of prostate cancer. Calcium, in high concentrations, has been found to inhibit the formation of 1,25(OH)2 vitamin D (active form of vitamin D). Therefore, it may lead to enhanced cellular proliferation in the prostate [79]. However, epidemiological studies did not find any relation between plasma levels of vitamin D and the risk of prostate cancer [79].

High intake of milk has been associated with increased serum levels of insulin-like growth factor-I (IGF-1) [80]. Increased circulating amounts of IGF-1 correlate with a higher risk of prostate cancer [81].

Alcoholic and Nonalcoholic Drinks

Alcohol is reported to increase the risk of a variety of cancers involving mouth [82], pharynx and larynx [83], esophagus [84], breast [85], stomach [86], lung [87], pancreas [88], liver [89], skin [90], kidney [91], and colorectum cancers [92].

The liver is responsible for alcohol (ethanol) metabolism, and alcohol can affect the liver's activity to metabolize nutrients, non-nutrient dietary component, as well as multiple hormones. Acetaldehyde is a toxic and carcinogenic metabolite of ethanol. Increased abuse of alcohol induces host cancer-developing mechanisms such as inflammation, oxidative stress, and lipid peroxidation [93]. Besides, it is hypothesized that alcohol acts as a solvent that increases the penetration of carcinogens such as tobacco components into cells, interrupts retinoid metabolism, and inhibits DNA repair mechanisms [94–96]. High consumption of alcohol is linked to malnutrition or poor dietary behaviors such as folate deficiency. This condition makes the host more prone to oncogenic effects of alcohol [97].

High and prolonged drinking of alcohol adversely affects the gut microbiome, which is associated with poor functioning of the gut barrier [98]. Thereby, the gut lumen will be exposed to higher levels of bacterial antigens such as lipopolysaccharide (LPS) and flagellin. Exposure to these bacterial products has been shown to enhance the risk of developing hepatocellular and colorectal cancers [99, 100]. For the explanation of the probable mechanism, it should be noted that conserved bacterial components such as LPS and flagellin can stimulate toll-like receptors (TLRs) and trigger inflammation, which is an important cancer-progressing mechanism.

Alcohol consumption is associated with increased levels of estrogen in the peripheral blood, whose role in the development of breast cancer is indisputable [101].

Additionally, alcohol may metabolize in mammary glands to acetaldehyde that its carcinogenic effect has been previously mentioned [102].

There are studies showing that drinking water contaminated with arsenic is associated with cancers of the urinary tract [103], skin [104], and lungs [105]. Arsenic and arsenic-derived metabolites facilitate cancer development through ROS causing disruption of the cell membrane and mitochondria, DNA damage, transcription factor dysfunction, and change in the expression of genes which are responsible for cell growth and survival [106, 107].

Tea contains abundant biologically active molecules such as polyphenols. There are animal model studies suggesting that green tea has a protective effect in bladder cancer development [108].

Coffee contains high levels of phenolic phytochemicals and natural diterpenes which exert

their anticancer effects via anti-oxidative and anti-inflammatory mechanisms, inhibition of DNA methylation [109], and induction of apoptosis [110, 111]. Coffee drinking has been reported to decrease the risk of cancers of the liver, mouth, pharynx, larynx, and skin (basal cell carcinoma and malignant melanoma) [112]. In addition, coffee is reported to decrease the risk of endometrium cancer. Consumption of coffee is linked to higher concentrations of sex hormone-binding globulin (SHBG) resulting in decreased bioavailable sex steroids in the blood [113–115]. Totally, a short luteal phase and low bioavailable sex steroids are associated with increased risk of endometrial cancer [113–116]. Coffee consumption is associated with low insulin levels [117]. Coffee can diminish the risk of developing endometrial cancer in an insulin-dependent manner. Adipose tissue secretes a number of biologically active molecules such as adiponectin. Coffee has been shown to increase circulating levels of adiponectin [114, 118]. High blood levels of adiponectin correlate with reduced risk of endometrial cancer [119, 120]. Adiponectin can induce apoptosis and exert anti-inflammatory and anti-angiogenic effects as well [118, 121].

Sugar-sweetened drinks, including sweetened waters, barley water, sodas, sports drinks, energy drinks, cordials, and tea- and coffee-based beverages, are increasingly consumed around the world. This can explain at least the partially increased prevalence of overweight and obesity and subsequently increased risk of cancer [122].

It is, thus, recommended to consume drinking water and unsweetened beverages and to not drink alcohol and sweetened drinks.

Fast Foods

Ease of access and acceptability of fast foods containing high amounts of fat, sugars, and starches are linked to universally elevated rates of overweight and obesity [123]. Consumption of processed meat and preserved vegetables is linked to elevated risk of cancers involving the stomach [124], pancreas [125], lung [126], esophagus [127], colorectum [128], and nasopharynx [51]. Preserved foods contain a high amount of salt. Animal studies have shown that salt can change the viscosity of mucus and augment the production of carcinogenic nitrosamines and N-nitroso chemicals [129]. Nitrosamines and their metabolites have been suggested to produce a cancer-developing effect [130]. Also, high salt consumption may result in colonization of *Helicobacter pylori*, which is the most significant risk factor for stomach cancer [131].

Like red meat, processed meats include high amounts of protein, hem iron, and fat which are underlying factors for cancer development [50]. Moreover, the fat content of processed meat is higher than in red meat, which results in higher secondary bile acids. Studies indicate the carcinogenic effect of secondary bile in gastrointestinal cancer [132].

In addition, processed meats such as sausages are exposed to high temperature during cooking procedure, which is associated with the higher production of heterocyclic amines and polycyclic aromatic hydrocarbons. Consumption of processed meats has been associated with increased insulin resistance and hyperinsulinemia which act as cancer-promoting factors [133].

Processed meats contain high amounts of exogenously derived N-nitroso compounds, nitrate, and nitrite, which have been implicated in tumorigenesis [134, 60].

Other Dietary Exposures

In general, it is believed that a weakened immune system and environmental toxins are responsible for cancer development [135]. Approximately 50% of cancer patients use some complementary and alternative medicine to strengthen their immune system and body detoxification from environmental pollution [136]. Therefore, a high proportion of cancer survivors follow a special anticancer diet or consume dietary supplements or both [137]. Cancer-fighting diet is recommended to patients with cancer. This includes raw vegetables and fruits, macrobiotics, alkaline diet, Gerson's regime, Budwig's regime, and low-carbohydrate or ketogenic diet. But there is yet no clinical evidence confirming the efficacy of such diet. Furthermore, there are data from clinical studies and case reports that reveal the harmfulness of such diet by increasing the risk of

malnutrition; weight loss; dehydration; metabolic acidosis; fatigue; sedation; anemia; deficiency in vitamins; calcium, iron, and zinc deficiency; hyponatremia; hyperkalemia; as well as hyperlipidemia [138].

Expert guidelines recommend patients with cancer to obtain nutrients from foods and to not consume supplements [139].

Nutritional insufficiency is endemic in many areas around the world, and supplementation is necessary to provide sufficient concentrations of nutrients in people with nutrient insufficiency.

Many clinical studies investigating the outcome of supplement intake in cancer survivors find no significant effect. However, some deleterious and advantageous effects are reported. For example, beta-carotene supplement is associated with increased risk of gastric and lung cancer, while vitamin E enhances mortality from colorectal and prostate cancers [140, 141]. However, both beta-carotene and vitamin E can decrease the toxicity of radiotherapy in patients with head and neck cancer while increasing the risk of cancer recurrence in smoker patients [142–145]. Selenium supplementation shows a dual function; its supplementation in selenium-deficient populations leads to reduced risk of stomach and lung cancer, while its consumption in persons with higher circulating levels of selenium increases the risk of these cancers [146]. Consumption of high-dose nutrition supplements is not recommended for cancer prevention.

In the absence of nutritional deficiency, people should achieve nutritional needs through diet alone. In addition, cancer clinicians should elucidate the potential adverse effects of supplements for cancer survivors, and in the case of necessity for supplementation, it should be supplied by a reliable source and personalized to the cancer patient by his/her physician.

Body Fatness

Experimental and epidemiological studies have shown that adult body fatness is associated with a higher risk of cancers affecting the head and neck [147], esophagus [148], liver [149], pancreas [150], colorectum [151], breast [152], kidney [153], stomach [154], gallbladder [155], endometrium [156], ovary [157], cervix [158], and prostate [159].

A number of underlying mechanisms are proposed for increased susceptibility to breast cancer in obese adults, which can also be extended to the other cancers.

Body fatness is associated with abnormal hormone profile, which plays an important role in carcinogenesis in their target sites. Obesity makes postmenopausal women more prone to invasive breast cancer. Increased risk of invasive breast cancer was evident among women with a BMI of more than 35 kg/m2 compared to women with a BMI of less than 25.0 kg/m2. In addition, mortality from breast cancer in women with grades 2 and 3 obesity was two times higher than that in nonobese women. Interestingly, normal body weight women who gained more than 5% of their body weight had increased risk of breast cancer compared to overweight or obese women who had no change in body weight [152]. During menopause, the breast tissue tends to have higher adipose content. After the decline of the production of estrogen from ovaries, breast tissue adipocytes are responsible for local estrogen production by conversion of androgens. Therefore, obese and overweight women have higher plasma concentration of estrogen that its role in the development of cancer is well-documented [160].

Additionally, obesity changes the adipocyte function in energy balance, whereby inflammation is increased and signaling of adipokine such as leptin and adiponectin [161] is changed. All these events are potential contributing factors to cancer [162, 163].

Obesity correlates with higher concentrations of circulating insulin, which, in turn, is associated with a greater risk of breast cancer [164]. Insulin by inhibiting the production of estrogen-binding protein makes estrogen more accessible for target tissues [165]. In addition, insulin can induce cancer development by supporting cell growth and preventing apoptosis [166, 167].

The common expectation is that fast body growth and breast tissue development in early life lead to DNA damage and cancer development. However, interestingly, obesity in early years shows a reverse association with the risk of breast

cancer in premenopausal and postmenopausal women. This association suggests a protective long-term effect of body fatness on the future risk of breast cancer. This association is controversial to the previously mentioned outcomes about the positive relationship between obesity and breast cancer risk in postmenopausal women. Abolished hormone profile in obese children and adolescents is responsible for reduced risk of cancer in adulthood.

Animal studies show that fat tissue-produced estrogen leads to earlier breast tissue development but also can reduce the susceptibility of breast tissue to carcinogenic agents [168]. Furthermore, young and obese girls are more prone to experience anovulation, linking to lower concentration of ovarian hormones and estradiol and diminished risk of breast cancer. Overweight and obese young people have lower blood concentration of IGF-1, which is the chief mediator of growth hormone [169]. Higher amounts of IGF-1 have been associated with increased risk of cancer [170]. Therefore, body fatness at a young age correlates to a reduced risk of cancer.

There are studies suggesting that greater body fatness may stimulate inflammation in the esophagus and thereby promote the progression of gastroesophageal reflux disease, which, in turn, may lead to the development of Barrett's esophagus. This condition may increase the risk of developing esophageal adenocarcinoma. Obesity is associated with increased risk of nonalcoholic fatty liver disease (NAFLD) that can adversely affect hepatic lipid metabolism. The severe form of NAFLD leads to oxidative stress and inflammation [171, 172], which are associated with liver cancer development.

Height and Birth Weight

Adult attained height is associated with increased risk of cancer involving the colorectum, prostate, lung [173], breast [174], endometrium [175], ovary [176], pancreas, kidney [177], and skin [178]. This might be due to the increased secretion of pituitary-derived growth hormone and insulin-like growth factors (IGFs) during the age of growth of taller people [179, 180]. Having more cells and increased number of cell divisions in taller individuals would result in increased risk of cancer development [181]. In addition, the increased length of the intestine in tall people seems to make them more exposed to mutagenic agents and therefore increased risk of colorectal cancer.

It is found that high birth weight is associated with increased risk of breast cancer [182] and malignant melanoma [183]. By an unknown mechanism, high birth weight pregnancies are linked to higher circulating levels of estriol and maybe increased activity of IGF-I [184, 185]. Also, additional measures at the birth time such as birth length, placental weight, and ponderal index are related to maternal blood levels of estrogen and breast cancer risk [184–189].

Physical Activity

Physical activity includes any work of skeletal muscles that consumes more energy than resting. Consequently, physical activity plays an important role in energy balance [139].

Physical activity influences a variety of immunologic, endocrine, and metabolic functions. Mostly, physical activity produces its anticancer effect through reducing body fatness. Body fat loss is associated with a reduction in plasma concentrations of estrogen, IGF-1, and fasting insulin as well as with insulin resistance and inflammation. The advantageous effect of physical activity on body fat reduction has been observed in cancers of the colorectum, breast, lung, liver, esophagus, and endometrium [164, 190–192].

Physically active persons revealed improvement in both the innate and adaptive immune responses and therefore in host tumor surveillance [193, 191]. Furthermore, physical activity is often linked to higher sunlight exposure and subsequently increased vitamin D absorption, which may prevent cancer development.

Lactation

Studies suggest a direct association between sex steroids and the risk of female malignancies such as breast, ovary, and endometrium cancers [194]. Lactation has been associated with lower risk of breast and ovarian cancers. The anticancer effect of breastfeeding is at least partially attributed to physiological amenorrhoea during pregnancy

and lactation, which suppress ovulation and thereby reduce host exposure to plasma estradiol [195–197].

The Role of Nutrition in Cancer Therapy

Research about nutrition and cancer is largely focused on investigating the effect of nutrients and dietary factors on cancer development. Many attempts have been made to design immune-enhancing diets for patients with cancer. Cytotoxicity during cancer chemotherapy can impair the nutritional status of patients and subsequently reduce the efficacy of antitumor treatments as well as lessen the quality of life in patients with cancer. In addition, there are studies that seek to establish standard immunonutrition for cancer patients who are undergoing surgery. Such a diet must be supplemented with high amounts of nutrients that can modulate immunological mechanisms such as glutamine; arginine; taurine; nucleotides; polyunsaturated fatty acids (omega-3); beta-carotene; vitamins A, E, and C; as well as trace elements including selenium and zinc, which support the host immune system to control inflammation and also stimulate protein synthesis [198–201].

Preoperative immunonutrition has been shown to correct the Th1/Th2 ratio in tumor-bearing and post-surgery states in patients with colorectal cancer [202]. Moreover, a randomized clinical trial on patients who had head and neck and esophageal cancer and received radiochemotherapy revealed that immunonutrition led to a sustained increase in the production of prostaglandin-E2, $CD4^+/CD8^+$ T-cells ratio and in the expression of CD3. Interestingly, immunonutrition is linked to increased expression of antioxidant enzymes, NADPH oxidase, as well as interleukin-6 receptor (IL-6r) and interleukin-10 receptor alpha (IL-10ra) [203]. Immunonutrition modulates immune cell responses affecting their phenotype. These alterations in the phenotype and abilities of immune cells make the host body capable of bearing oxidative stress and inflammation, which are caused by radiochemotherapy.

The efficacy of pre- and postoperative immunonutrition has been frequently studied in the surgeries of gastrointestinal tumors [204, 205, 198, 199]. Immunonutrition has been found to improve surgical outcome. In addition, it can be a cost-effective approach by reducing infectious complication and hospitalization days [206, 207]. It seems that preoperative use of immunonutrition is more efficient because its administration in the preoperation phase provides timely and adequate circulating levels of immunonutrients at the beginning of the postoperative phase, which then help in controlling inflammation [206]. However, the preoperative administration of immunonutrition has been shown to be less effective in malnourished patients with cancer [208].

Conclusions

There are many data indicating the substantial role of diet and nutritional ingredients in the prevention of cancer as well as their potential for application in therapeutic regimens for patients diagnosed with cancer.

It is clear that nutritional recommendations for cancer prevention require an evidence-based knowledge about causes of cancer and the nutritional behavior patterns of that population. It should be noted that our understanding of the context is largely based on animal and human studies, which are not comprehensive or systematic research works. However, there is increasing data linking nutrition and cancer can provide preventive and therapeutic approaches to diminish the risk of cancer and its related burden.

In 2018, the World Cancer Research Fund (WCRF) and American Institute for Cancer Research (AICR) provided the general recommendations on cancer prevention and survival according to recent findings of diet, nutrition, and physical activity.

People with lower socioeconomic status are more likely to be exposed to cancer risk factors. Ministries' joint activities, public health administration, and public agencies are necessary to create an environment that is able to make people eager to consume a diet rich in whole grains,

non-starchy vegetables, and fruits. Additionally, it is essential that policymakers provide backgrounds to motivate the society to change their sedentary lifestyle and to limit consumption of fast foods, alcohol, and sugar-sweetened drinks as well as other processed foods high in fat, starches, or sugars. Governments are responsible for monitoring and controlling cancer risk factors especially those that people cannot necessarily affect them, for example contamination of drinking water by arsenic.

A broad set of policies is essential to support breastfeeding such as promoting breastfeeding in hospitals, offering free consultations in healthcare centers, workplace regulations, as well as marketing regulations of breast milk substitutes.

The recent advances in genetics, epigenetics, metabolomics, immunology, dietary metabolite biomarkers, as well as available data from systematic studies and computer analysis of intricate exposures lead to increase our knowledge about intricacies of the influence of nutrition on cancer. In spite of advances in the area of nutrition, immunity, and cancer, comprehensive studies are needed to exactly unravel the association between nutrients and immunological pathways contributing to cancer especially according to ethnic, age, sex, and environmental exposures such as smoking, UV light radiation, and infections. Future studies should investigate the optimal dose of pre- and postoperative immunonutrition to increase the efficacy of cancer surgery. These studies should evaluate the effectiveness of immunonutrition during neoadjuvant therapies as well.

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14

Aging, Immunity, and Neuroinflammation: The Modulatory Potential of Nutrition

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Contents

Key Points

- The changes in the immune system that accompany human aging are very complex and are generally referred to as immunosenescence.
- The process of aging is commonly accompanied by low-grade inflammation thought to contribute to neuroinflammation and to many age-related diseases.
- Several lifestyle strategies, such as intervening to provide an adequate diet and physical and mental activity, have been shown to result in improved immune and neuroprotective functions, a decrease in oxidative stress and inflammation, and a potential increase in individual longevity.

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Introduction

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), physical activity, mental well-being, as well as gender and ethnicity, genetic background, psychosocial parameters (including stress), socioeconomic status, early-life events, and different chronic infections [1, 2].

Aging represents a major nutritional challenge, not only concerning the dietary supply of certain nutrients but also in terms of their altered metabolism [3]. Macro- and micronutrient deficiencies, which are very common in the elderly, have been found to be associated with a physiological decline in various body functions, which can lead to higher morbidity and mortality [2]. Nutrient status represents an important factor

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contributing to immune competence, since undernutrition impairs the immune system, suppressing immune functions that are fundamental to host protection. Undernutrition leading to an impairment of immune function can be due either to insufficient intake of energy and macronutrients or to specific deficiencies in some particular micronutrients, although often these occur in combination [4].

Among other micronutrients, zinc, for instance, has an essential significance to health; its deficiency is responsible for various diseases. Zinc is one of the most important trace elements in the organism, with three major biological roles, as a catalyst, structural, and regulatory ion. It plays a critical role in organism homeostasis, in immune function, in oxidative stress, in apoptosis, and in other physiological activities [2, 5]. Thus, zinc deficiency may adversely affect the immunological status, increase oxidative stress, lead to the generation of inflammatory cytokines, and influence the progression of many chronic diseases, including atherosclerosis, neurological disorders, autoimmune diseases, age-related degenerative diseases, and various malignancies [5]. Zinc deficiency is known to decrease innate immune function. It particularly impairs the lytic activity of natural killer (NK) cells, reduces natural killer T (NKT)-cell cytotoxicity and immune signaling, influences the neuroendocrine-immune pathway, and alters cytokine generation in mast cells [6, 7].

However, excessive amounts of some nutrients may also impair immune function. Overnutrition, combined with an inactive lifestyle and sedentary behavior, promotes the accumulation of visceral fat and leads to obesity. Accumulating evidence indicates that obesity causes chronic low-grade inflammation and the development of systemic metabolic dysfunction that appears to be aetiologically associated with obesity-linked disorders [2, 8]. Adipose tissue acts as a key endocrine organ by releasing bioactive substances, known as adipokines, that may have either pro- or anti-inflammatory activities [9]. The production of pro-inflammatory adipokines, such as tumor necrosis factor (TNF), leptin, retinol-binding protein 4, lipocalin 2, IL-6, IL-18, and angiopoietin-like protein 2, in expanding fat tissue increases, while the concentrations of anti-inflammatory cytokines are reduced [9]. This process is accompanied by an infiltration of adipose tissue with pro-inflammatory mediators and the induction of a low-grade inflammatory state, which is characterized by elevated levels of circulating inflammation markers, such as IL-6, TNF, and C-reactive protein (CRP). Adipose tissue is infiltrated with macrophages in two separate polarization states: M1, which produces pro-inflammatory cytokines, and M2, which produces anti-inflammatory cytokines. Therefore, it has been proposed that in the adipose tissue, a phenotypic switch takes place toward the macrophages of M1-phenotype, promoting the inflammatory state. This low-grade systemic inflammation is known to be associated with the development of atherosclerosis, neurodegeneration, insulin resistance, and the promotion of tumor growth [10]. It was also demonstrated that diet-induced obesity recruits monocytes from the periphery to the brain following herpes simplex virus (HSV)-1 latency in mice [11], leading to an exaggerated neuroinflammatory response and the promotion of neurodegeneration. While these phenomena are not limited to the elderly, they often tend to be exacerbated in older people who, for one reason or another, exercise less than the young [2].

Thus, many chronic and neurodegenerative diseases can be prevented by changing lifestyle and behavioral habits, particularly dietary habits, and exercise. For example, a positive effect of a 3-month regimen of comprehensive lifestyle changes (plant-based diet, moderate exercise, stress management, and improved social support) on increased telomerase activity was demonstrated in men with low-risk prostate cancer. After a 5-year follow-up, relative telomere lengths of lymphocytes were increased in the lifestyle intervention group and decreased in the control group [12]. According to the findings of another study on more than 23,000 adults, a healthy lifestyle alone lowered the risk of developing chronic diseases with known inflammatory etiology by 78% [13]. Although such changes are thought to be beneficial, more investigations are

needed to confirm whether this is really the case, and the full biological implications remain to be determined in large RCTs [2, 14].

Thus, recent studies indicate the need for a more in-depth, holistic approach to determine the optimal nutritional and behavioral strategies that would maintain immune and other physiological systems in the elderly people. Superimposed on chronological age alone, the remodeling of immunity as a result of interactions with the environment over the life course is instrumental in shaping immune status in later life. In addition to interactions with pathogens, host microbiome and nutrition, exercise and stress, and many other extrinsic factors are crucial modulators of this immunosenescence process [2]. In the next sections, we briefly describe the observed age-related changes in the immune system and then outline the possible contribution of inflammaging and immunosenescence to neuroinflammation and finally discuss the modulatory potential of nutrition and active lifestyle thereon.

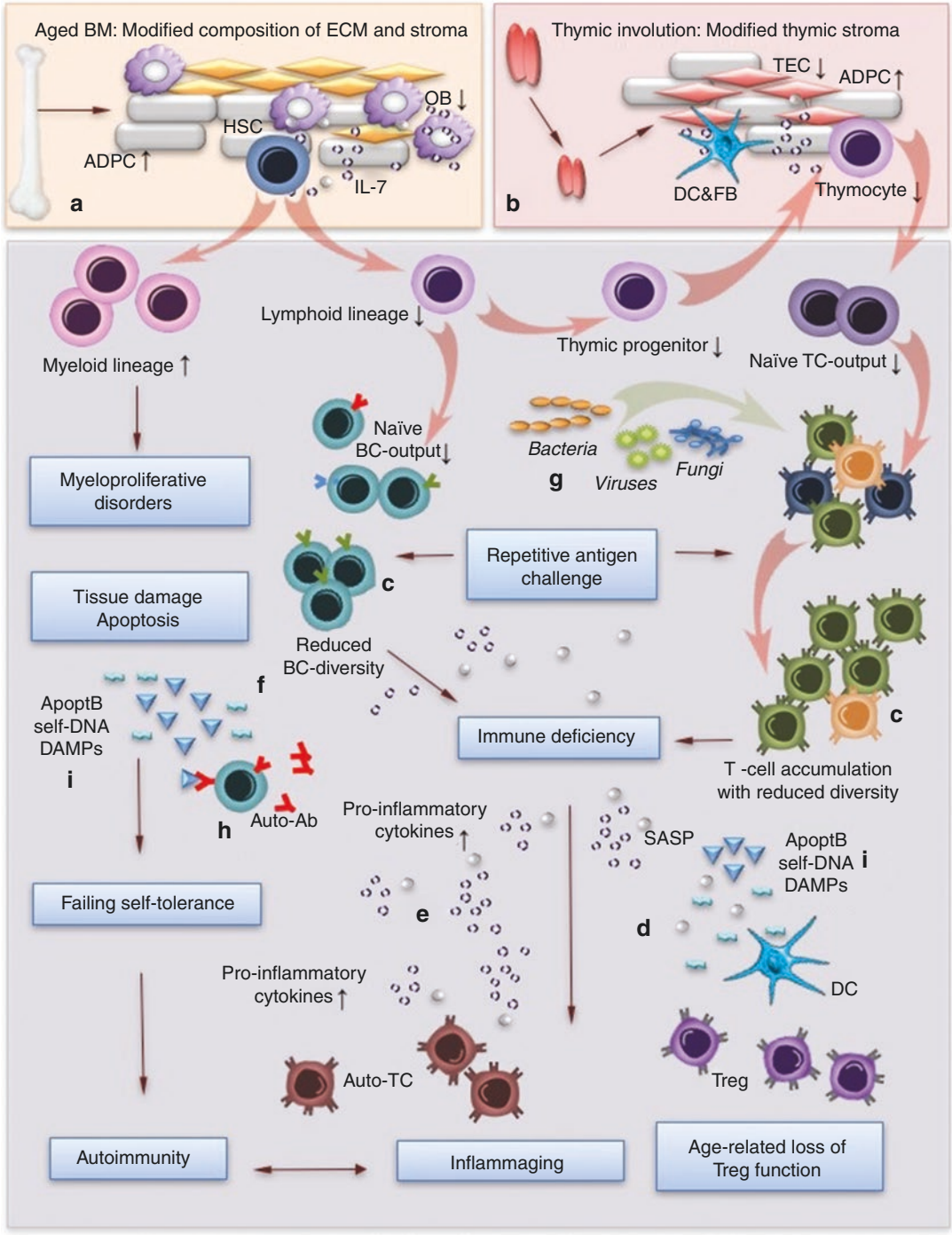
Immunosenescence and Inflammaging

Human immune aging represents a universal and multifaceted process characterized by progressive immunodeficiency, chronic inflammation, and autoimmunity [15, 16]. The complex regulatory circuits required for maintaining appropriate physiological homeostasis may become compromised with aging [17, 18]. Age-related immune dysfunction (in combination with other mechanisms) may at least in part explain the process of aging itself [19], as originally proposed by Walford in 1969 [20].

In the course of aging, our immune system undergoes an imprecisely defined process of immunosenescence that affects both adaptive and innate immune systems. The most marked changes in adaptive immunity are decreased numbers of peripheral naïve T cells and the concomitant accumulation of late-stage differentiated memory T cells [18, 21–28] with reduced antigen receptor repertoire diversity [26, 29–31] (Figs. 14.1 and 14.2). This phenomenon results from poorly understood age-related impairments in the hematopoietic stem cell (HSC) compartment which generates fewer T-cell precursors in adult and later life, on the one hand, and thymic involution at puberty which markedly reduces the production of mature T cells from their precursors, on the other hand [32].

The HSC compartment (Fig. 14.1) is negatively modulated and functionally affected by aging [33] and is increasingly substituted by adipose tissue [18, 34, 35]. Age-related modification within the HSC compartment may be partly due to the "intrinsic cellular aging" of HSCs themselves. Aged HSCs demonstrate more accumulated DNA damage, telomere attrition, and epigenetic deregulation, often in combination with an increase in intracellular reactive oxygen species (ROS) [2, 36]. Such events may also lead to progressive myeloproliferative disorders and the malignant transformation of HSCs. In addition to such "intrinsic" HSC age effects, the extracellular and stromal matrix of the aging bone marrow (which normally nurtures and drives stem cell production) also undergoes dramatic structural changes in terms of the numbers of stromal cells and osteoblasts and reduced production of IL-7 (Fig. 14.1). The aged pool of HSCs is often characterized by a marked shift in lymphoid and myeloid lineage output. Such age-associated myeloid skewing of the differentiation potential, together with decreased homing efficiency, contributes to changes in the cellular composition of the HSC compartment and is believed to be an important contributor to the decline of immune competence in the elderly [2, 37, 38].

Given the age-associated alterations at the level of immune cell production in the bone marrow and the skewing toward myeloid differentiation in the cells exported to the periphery, it is not surprising that immune functioning also changes with age. Not only are fewer B cells produced and fewer T-cell progenitors, but in the case of the latter, their development in the thymus is compromised by the universal process of thymic involution (Fig. 14.1). Although this may be viewed as a developmental event and not truly a senescence effect, it is thought to have dramatic consequences for immunity over the lifespan [18].


Aged BM: Modified composition of ECM and stroma
OB ↓
HSC
ADPC ↑
IL-7
a
Thymic involution: Modified thymic stroma
TEC ↓
ADPC ↑
DC&FB
Thymocyte ↓
b
Myeloid lineage ↑
Lymphoid lineage ↓
Thymic progenitor ↓
Naïve TC-output ↓
Naïve BC-output ↓
Bacteria
Viruses
Fungi
g
Myeloproliferative disorders
Repetitive antigen challenge
c
Tissue damage Apoptosis
Reduced BC-diversity
f
ApoptB self-DNA DAMPs
i
Immune deficiency
T -cell accumulation with reduced diversity
h
Auto-Ab
Pro-inflammatory cytokines ↑
SASP
ApoptB self-DNA DAMPs
i
Failing self-tolerance
e
d
DC
Pro-inflammatory cytokines ↑
Auto-TC
Treg
Autoimmunity
Inflammaging
Age-related loss of Treg function

Both extrinsic and intrinsic factors are considered (Fig. 14.1) to be involved in causing thymic involution, including the thymic microenvironment as well as the impaired development of aged thymocytes themselves [39]. During this ongoing process, beginning even before puberty, as in the bone marrow, a progressive replacement of lymphoid tissue by fatty tissue takes place, accompanied by a reduction of the active areas of thymopoiesis [40]. Most elderly people do retain some residual thymic function, which may act to provide a continuous low-level input of new naïve T cells (Fig. 14.1) to the periphery [41] at least until extreme old age [18]. The end result of thymic involution is that the individual is equipped with a set of naïve T cells early in life, but which are not constantly replaced at the same level throughout life. Thus, elderly people have vanishingly small numbers of naïve T cells and accumulations of memory T cells (Figs. 14.1 and 14.2), mostly specific for the pathogens that they have previously encountered [18].

Aged individuals have decreased protection against newly arising infections as well as difficulties in controlling endogenous persistent viral infections [42]. According to a current paradigm, the age-associated shrinkage of compartment size and diminution in T- and B-cell receptor diversity (Fig. 14.1) appears to be responsible for the impaired protective ability of the immune system to respond to a universe of antigens [25, 26, 42]. Particularly, dramatic changes with age are seen in the repertoire diversity that declines dramatically [29, 31].

Lifelong exposure to different pathogens (Figs. 14.1 and 14.2) is regarded as a major driving factor of the phenotypic changes in the distribution of T-cell subsets over the life course. For unclear reasons, especially a latent infection with cytomegalovirus (CMV), but not with any other herpes viruses, with recurrent episodes of reactivation has been found to promote memory T-cell "inflation" and drive T cells to a late stage of differentiation. In aged individuals, oligoclonally expanded $CD8^+$ T cells show an increased expression of late-stage differentiation markers. The accumulation of these highly differentiated T cells, at least some of which may be truly senescent, contributes to the age-associated increased production of pro-inflammatory cytokines and low-grade inflammation and could thus possibly also contribute to age-related morbidity and mortality [24, 43–47].

Regulatory T cells (Tregs) play a central role in immune regulation (Fig. 14.1), providing a delicate balance between protective and patho-

Fig. 14.1 The aging immune system. The HSC compartment is negatively modulated and functionally affected by aging (**a**). Age-related modification within the HSC compartment may be partly due to "intrinsic cellular aging" of HSCs themselves, with accumulated DNA damage, telomere attrition, and epigenetic deregulation. The extracellular and stromal matrix also undergoes dramatic structural changes in terms of the numbers of stromal cells and osteoblasts and the reduced production of IL-7. The aged pool of HSC shows a marked shift in lymphoid and myeloid lineage output. Thymic involution (**b**) involves both extrinsic and intrinsic factors, including the thymic microenvironment as well as the impaired development of aged thymocytes themselves. A progressive replacement of lymphoid tissue by fatty tissue takes place, accompanied by a reduction of the active areas of thymopoiesis. The end result of thymic involution is vanishingly small numbers of naïve TC and accumulations of memory TC (**c**), mostly specific for the pathogens that they have previously encountered. Age-affected Tregs (**d**) can induce chronic inflammation, an increased risk of autoimmunity, but also be responsible for diminished immunity. Elevated inflammatory immune mediators, SARS (**e**), are responsible for the activation of self-reactive memory BC, producing increased levels of auto-Ab (**f**), which are frequent in aged individuals as a result of tissue damage and apoptosis. Repetitive antigenic challenges over the lifetime (**g**) may stimulate pro-inflammatory cytokines, contributing to the maintenance of persistent chronic inflammation. Self-reactive B and T cells (**h**) recognize a self-antigen and differentiate into memory and effector cells due to a breakdown of the control of autoreactivity. Chronic inflammation additionally resulting in tissue injury and apoptosis attracts DCs, which in elderly people have impaired the ability to take up apoptotic cells (**i**). Instead, they induce a vicious circle with a further pro-inflammatory response and increased reactivity to self-DNA, which may be more immunogenic in the elderly. Additionally, DAMPs (I), present in proteins released during cell necrosis, promote sterile inflammation. ECM, extracellular matrix; HSC, hematopoietic stem cell; TEC, thymic epithelial cells; ADPC, adipocytes; OB, osteoblasts; DC, dendritic cells; FB, fibroblasts; BM, bone marrow; BC, B cells; TC, T cells; auto-TC, autoreactive T cells; Tregs, regulatory TC; MDSCs, myeloid-derived suppressor cells; ApoptB, apoptotic bodies; DNA, deoxyribonucleic acid; DAMPs, damage-associated molecular patterns; SASP, senescence-associated secretory phenotype; IL, interleukin; Auto-Ab, autoantibodies. (Modified from Müller and Pawelec [18])

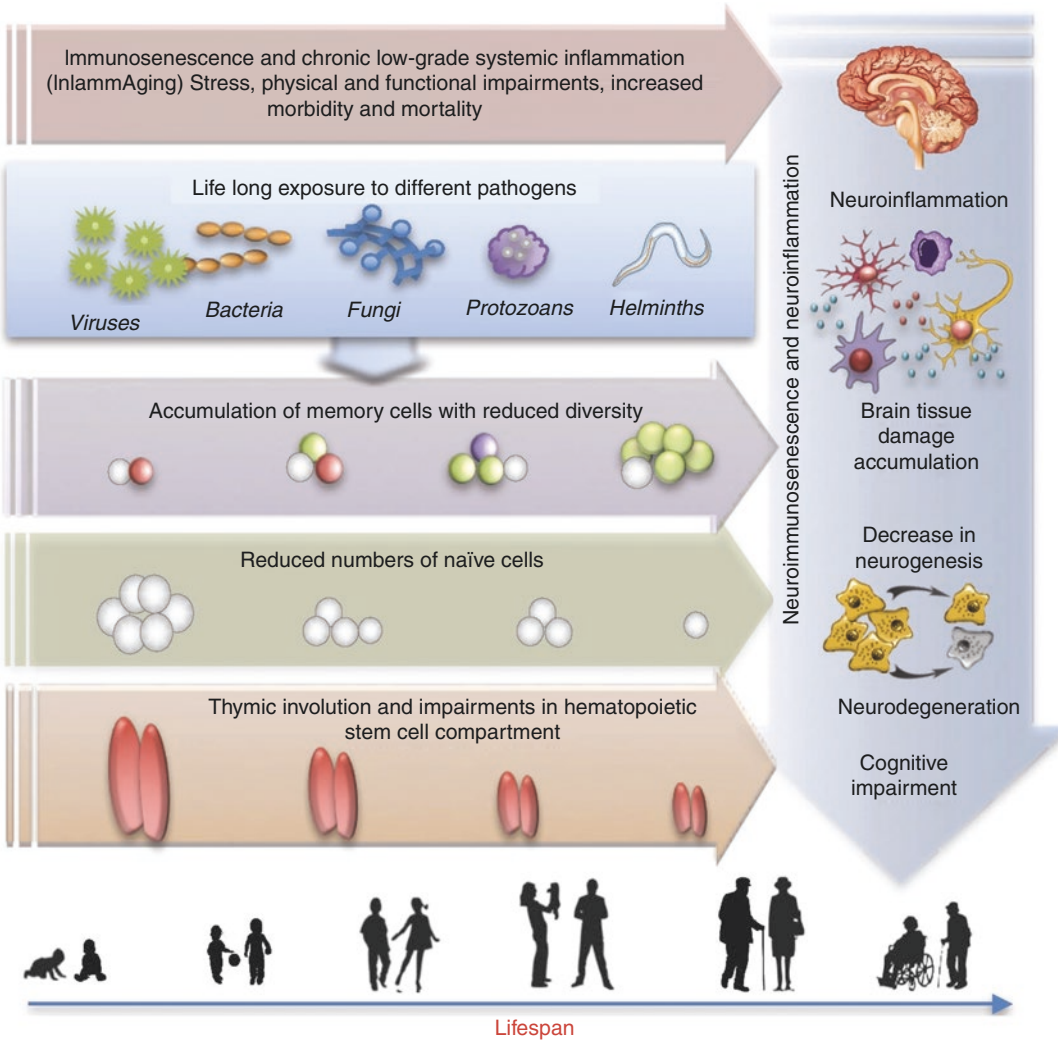


Fig. 14.2 Potential mechanisms of immunosenescence and neurosenescence. Immunosenescence affects both adaptive and innate immune systems. The most relevant changes to adaptive immunity are decreased peripheral naïve T cells and the concomitant accumulation of late-stage differentiated memory T cells with reduced antigen receptor repertoire diversity. This phenomenon results from age-related impairments in the hematopoietic stem cell compartment and thymic involution. Lifelong exposure to different pathogens is the major driver of the phenotypic changes in the distribution of T-cell subsets over the life course. Aging is characterized by a chronic, low-grade inflammation (inflammaging). Peripheral immunosenescence and inflammaging may promote neuroinflammation by modulating glial cells toward a more active pro-inflammatory state, leading to a loss of neuroprotective function, to neuronal dysfunction accumulation of brain tissue damage, and to neurodegeneration. (Modified from Di Benedetto et al. [65]. https://doi.org/10.1016/j.neubiorev.2017.01.044)

genic immune responses [18]. Early data indicated that younger and older people possessed phenotypically identical Tregs but that suppressive function waned in the latter [48]. More recent data confirm that aging significantly affects the generation, homeostasis, diversity, and functional competence of Tregs. A decline of thymic function disturbs the thymic Treg production and also seems to be compensated for through the peripheral expansion of

pre-existing Tregs and/or through the conversion of conventional T cells into Tregs [49], as had been demonstrated using T-cell clonal culture models much earlier [50]. Thus, due to the leading role of Tregs in immune homeostasis, an age-related deterioration of Tregs (Fig. 14.1) might induce either excessive immunity (impacting a chronic inflammation as well as increasing the risk for autoimmunity) or, conversely, an age-related increase of Treg activity might result in failing immunity (raising the risk of malignancies and infectious diseases) in the elderly [49, 51, 52].

As we age, the maintenance of homeostatic conditions may become compromised [18]. The consequence of this imbalance may be that immunity is redirected toward the innate immune responses, characterized by increased levels of systemic inflammatory molecules – a phenomenon dubbed as inflammaging [44]. Large epidemiologic studies commonly report about elevated levels of inflammatory factors such as CRP, IL-6, and TNF in the peripheral blood of elderly people [53].

Elevated inflammatory immune mediators (Fig. 14.1) are thought to be involved in most age-related chronic and neurodegenerative diseases as well as carcinogenesis. Over the life course, inflammatory stimuli from pathogen sources are likely to become increasingly entangled with endogenous stimuli. One possible source of this endogenous "noise" is the senescence-associated secretory phenotype (SASP) characterizing the accumulating "senescent" cells in the elderly that can affect the behavior of neighboring cells [54, 55]. The SASP is a spectrum of pro-inflammatory proteins and cytokines secreted by such cells, which have permanently exited the cell cycle and are known to accumulate in the periphery to significant numbers with advancing age [56, 57].

This process is also associated with the activation of self-reactive memory B cells, producing increased levels of autoantibodies (Fig. 14.1), which are frequent in aged individuals as a result of tissue damage and apoptosis [58]. Repetitive antigenic challenges over a lifetime (Figs. 14.1 and 14.2) may stimulate pro-inflammatory cytokines, contributing to the maintenance of persistent chronic inflammation [59]. Such persistent age-related chronic inflammation represents an extreme stress factor for the immune system, causing eventual exhaustion. Autoimmunity is associated with what could be considered premature immune aging, demonstrating the relationship between chronic immune stimulation and progressive immunosenescence [18, 60].

According to the current paradigm, autoimmunity develops when self-reactive B and T cells (Fig. 14.1) recognize a self-antigen and differentiate into memory and effector cells due to the breakdown of the control of autoreactivity [15]. It can occur by a selection of T cells with an elevated affinity for self-antigens or latent viruses, which are at the same time also pro-inflammatory. The chronic inflammation additionally resulting in tissue injury and apoptosis attracts dendritic cells (DC), which in elderly people have an impaired ability to take up apoptotic cells (Fig. 14.1). Instead, they induce a vicious circle with a further pro-inflammatory response and increased reactivity to self-DNA, which may be more immunogenic in the elderly due to increased hypomethylation [16]. Additionally, damage-associated molecular patterns (DAMPs), present on proteins released during cell necrosis and promoting sterile inflammation, also become more frequent with advancing age [17, 18].

It has been suggested that aging is associated with chronic innate immune activation and significant changes in the functions of monocytes and macrophages, which may have implications for increased low-grade chronic inflammation and for the development of age-related diseases [61]. Monocytes are known to be mediators of the inflammatory response and comprise at least three different subsets, namely, classical, intermediate, and nonclassical monocytes. An age-related increase in frequencies of intermediate and nonclassical monocytes has been reported [62, 61]. Our results from the Berlin Aging Study II confirmed these findings, where we also found an age-related increase in frequencies of intermediate and nonclassical monocytes [63]. Together with

age-related macrophage activation, inflammatory monocytes contribute to the subclinical chronic inflammatory process [44, 64, 65].

The immune and central nervous systems represent two adaptive physiological systems of the body, which extensively communicate with each other throughout the lifespan. Given the multifaceted interactions between these systems and their tight interdependency, it is to be expected that peripheral immunosenescence and inflammaging contribute to neuroinflammation. Peripheral low-grade inflammation may promote inflammatory processes in the aged brain (Fig. 14.2) by modulating glial cells toward a more active pro-inflammatory state, leading to the loss of neuroprotective function, to neuronal dysfunction, and to the accumulation of brain tissue damage [65–67]. Systemic inflammation may, therefore, increase the risk of developing cognitive impairment, neurological disorders, and neurodegeneration [68–70]. In the next section, we will have a closer look at how inflammaging may contribute to the process of neuroinflammation during aging.

Neuroinflammation and the Aging Brain

Human aging is characterized by an impairment of cognitive abilities. Although no agreement exists on the basic mechanisms involved in this process, neuroinflammation appears to be the main contributor that links together many factors associated with cognitive aging [65, 71]. As we age, we experience greater susceptibility to memory impairments following an immune challenge that is characterized by an increased and prolonged production of pro-inflammatory cytokines in the otherwise healthy aged brain [65, 72]. It is widely established that both aging and stress can affect the neuroendocrine system, activate the hypothalamic-pituitary-adrenal (HPA) axis to release corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, and cause the anterior pituitary gland to secrete adrenocorticotropin (ACTH) (Fig. 14.3). This, in turn, induces the release of glucocorticoids from the adrenal gland into the circulation [73]. Cortisol affects the immune system in different ways by regulating the expression of cytokines [74], chemokines, and adhesion molecules and by affecting immune cell migration, maturation, and differentiation [65, 72, 75]. High levels of cortisol can negatively influence hippocampal neurogenesis directly or indirectly through the regulation of the expression of cytokines and their receptors on the brain and immune cells.

Recent ultrastructural analyses have uncovered a new player in the age-related remodeling of neuronal circuits, especially at the synapse level, that is rare under homeostatic conditions but becomes abundant during aging, neurodegeneration, and chronic stress [76]. These hyperactive "dark microglia" (Fig. 14.3) frequently reach into synaptic clefts with their highly ramified and thin processes, extensively encircle axon terminals and dendritic spines, and engulf them [76, 77]. Aging and neurodegeneration are characterized by dysregulated interactions with synapses, resulting in neuronal loss, which, in turn, represents the best pathological correlate of cognitive decline [77]. These conditions can sensitize the aged brain to produce an exaggerated response following exposure to a stressor or to the presence of an immune stimulus in the periphery [78]. Altered microglia profiles together with impairments in key regulatory systems can lead to prolonged neuroinflammation and age-related neurobehavioral complications [65, 79].

On the systemic level, peripheral immunosenescence and inflammaging lead to age-related changes in the blood (Fig. 14.3) [65]. Chronic exposure to inflammatory mediators may disrupt the endothelial barrier and allow for the transfer of immune cells and numerous pro-inflammatory cytokines into the brain parenchyma that, in turn, can modulate microglial phenotype and reactivity and drive low-grade brain inflammation. Brain cells, such as microglia, astrocytes, and neurons, as well as peripheral immune cells, such as T cells, monocytes, and macrophages, participate in inflammation (Fig. 14.3). It would provide an inflammatory milieu that is populated by all these resident and additional infiltrating immune cells that participate in a complex inter-

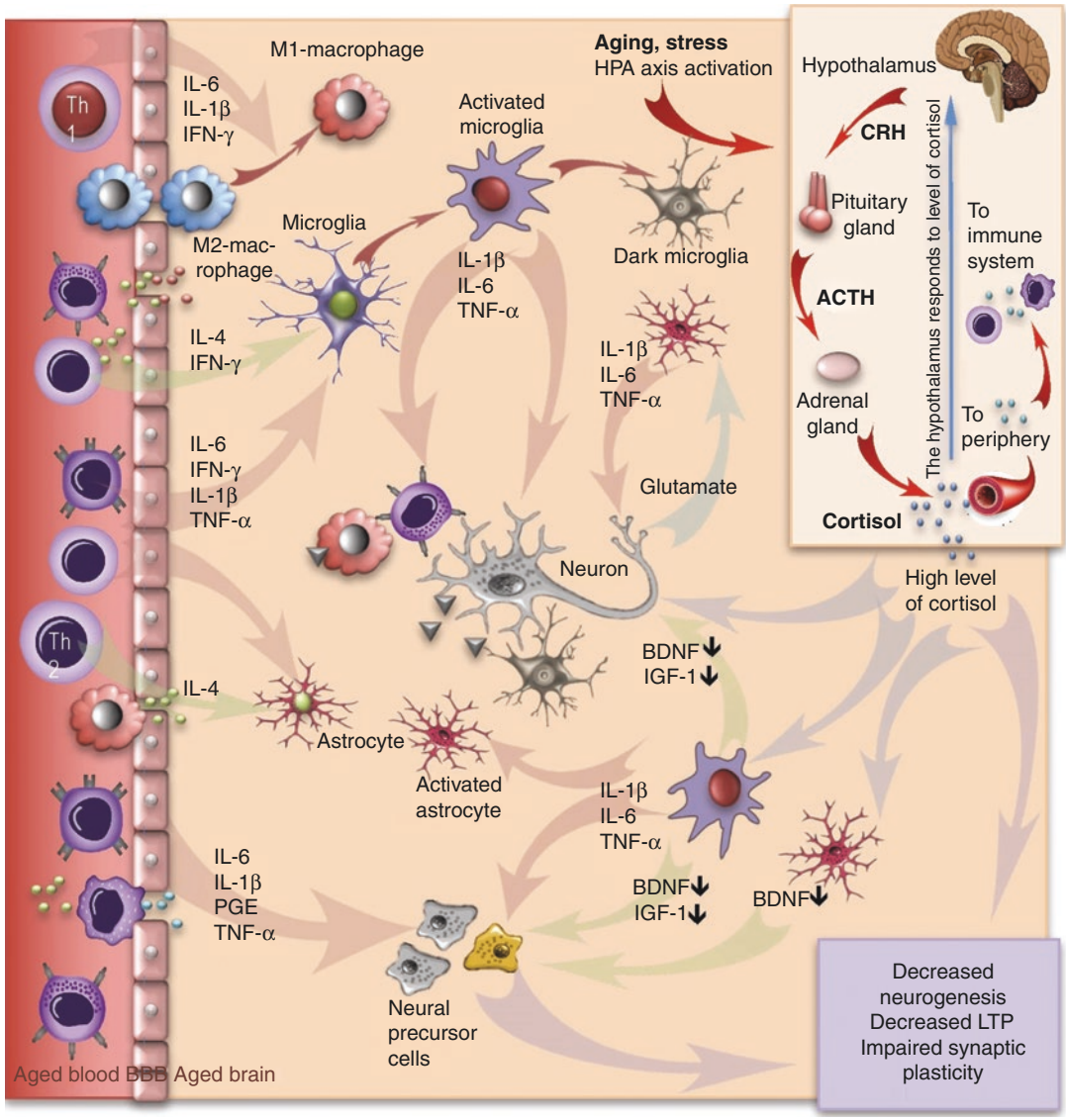


Fig. 14.3 The aging brain and neuroinflammation. Aging and stress activate the HPA axis to release CRH from the paraventricular nucleus of the hypothalamus and cause the anterior pituitary gland to secrete ACTH. This, in turn, induces the release of glucocorticoids from the adrenal gland into the circulation. High levels of cortisol can negatively influence hippocampal neurogenesis directly or indirectly through the regulation of an expression of cytokines and their receptors on the brain and immune cells. Peripheral immunosenescence and inflammaging lead to age-related changes in the blood. Chronic exposure to inflammatory mediators may disrupt the endothelial barrier and allow for the transfer of immune cells and numerous pro-inflammatory cytokines into the brain parenchyma that, in turn, can modulate microglial phenotype and reactivity and drive low-grade brain inflammation. Activated microglia and astrocytes change their morphology and function and produce pro-inflammatory cytokines, such as IL-1 β, IL-6, and TNF. Macrophages change their protective M2-phenotype to the pro-inflammatory M1-phenotype, thus contributing to further neuroinflammation. This overproduction of pro-inflammatory mediators disrupts the delicate balance needed for LTP induction, impairs synaptic plasticity, and reduces the production of BDNF and IGF-1, thus having detrimental consequences for neural precursor cells as well as for the normal neuronal functioning. HPA, hypothalamic-pituitary-adrenal axis; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; LTP, long-term memory potentiation; BDNF, brain-derived neurotrophic factor; IGF, insulin-like growth factor. (Modified from Di Benedetto et al. [65]. https://doi.org/10.1016/j.neubiorev.2017.01.044)

play between secreted inflammatory modulators and activated cell surface receptors, such as Toll-like receptors (TLRs) [65]. These receptors are primarily expressed on cells that play central roles in inflammatory response, including macrophages and microglia [80].

Activated microglia and astrocytes change their morphology and function and produce pro-inflammatory cytokines such as IL-1β, IL-6, and TNF [65]. Recent work suggests that microglia undergo a process of senescence, similar to the one in peripheral immune cells. Senescent and hyperactive microglia have been detected in the aged and diseased brain [81]. The aging brain, in turn, is apparently able to modulate the immune system and to support the recruitment of immune cells from the periphery, thereby contributing further to immunosenescence and neuroinflammation [82]. In addition, macrophages change their protective M2-phenotype to the pro-inflammatory M1-phenotype, thus contributing to further neuroinflammation [65]. As the aged brain is already "primed" to respond to inflammatory stimuli, additional stress or infection may induce more detrimental changes in the cognitive functioning of aged individuals [78, 83]. Furthermore, the age-related concurrent reduction of anti-inflammatory molecules also contributes to a sensitization to extrinsic and intrinsic stressors. T cells produce less IL-4 and IL-10 and more interferon gamma (IFN-γ), thereby promoting microglial activation. This has detrimental consequences for neural precursor cells, leading to a decrease of neurogenesis (Fig. 14.3) as well as increased decrements in learning and memory [84, 85].

Taken together, the overproduction of pro-inflammatory mediators in the periphery may induce a progressive increase in neuroinflammation, characterized by increased glial activation, elevated steady-state levels of inflammatory cytokines, and a decreased production of anti-inflammatory molecules, even in neurologically intact aged individuals [65]. In the next section, we consider the modulatory potential of nutrition in its ability, in combination with physical activity, to mediate anti-inflammatory effects and thus positively influence immunity and the aging brain.

The Impact of Nutrition and Physical Activity

Evidence that long-term behavioral changes, including nutritional intervention and reduced energy intake together with physical activity, may prevent, improve, or even reverse age-related impairments in immune function continues to accumulate [2]. Lifestyle factors, such as diet and exercise, have been established as playing an important role in immunosenescence, and the practice of "healthy" behavior may minimize the age-associated decline of immune function (Figs. 14.4 and 14.5).

Accumulating data strongly suggest that inflammation and oxidative stress are the main inducers of cellular aging (Fig. 14.5). The cross talk between oxidative stress and inflammation is a complex process, and there are studies reporting that ROS can stimulate inflammation via the activation of inflammasomes and the production of cytokines such as IL-1β and IL-18, which subsequently trigger inflammatory responses [86].

At least in the context of adiposity and inflammation mentioned above, as well as via multiple other postulated physiological effects, caloric restriction (CR) in humans might have beneficial effects in terms of lowering metabolism, a reduction of visceral fat, and weight loss [2]. CR has been shown to delay signs of immunosenescence in animals and is considered today as the only known method to prolong median as well as maximal lifespan in several tested species, from invertebrates to rodents and even including non-human primates [32, 87]. Thus, in rodents and nonhuman primates, CR leads to an attenuation of the age-related shift from naïve to memory-phenotype T cells and maintains a higher number of naïve T cells in aged animals [88]. Furthermore, the age-associated rise of pro-inflammatory cytokines, such as IL-6, IFN-γ, and TNF, and the resulting pro-inflammatory state of an aged immune system can be reversed by CR. It has been reported that the age-related decrease in the proliferative capacity of T cells (due to the shift from naïve to memory-phenotype T cells) can be reversed by CR [2, 32].

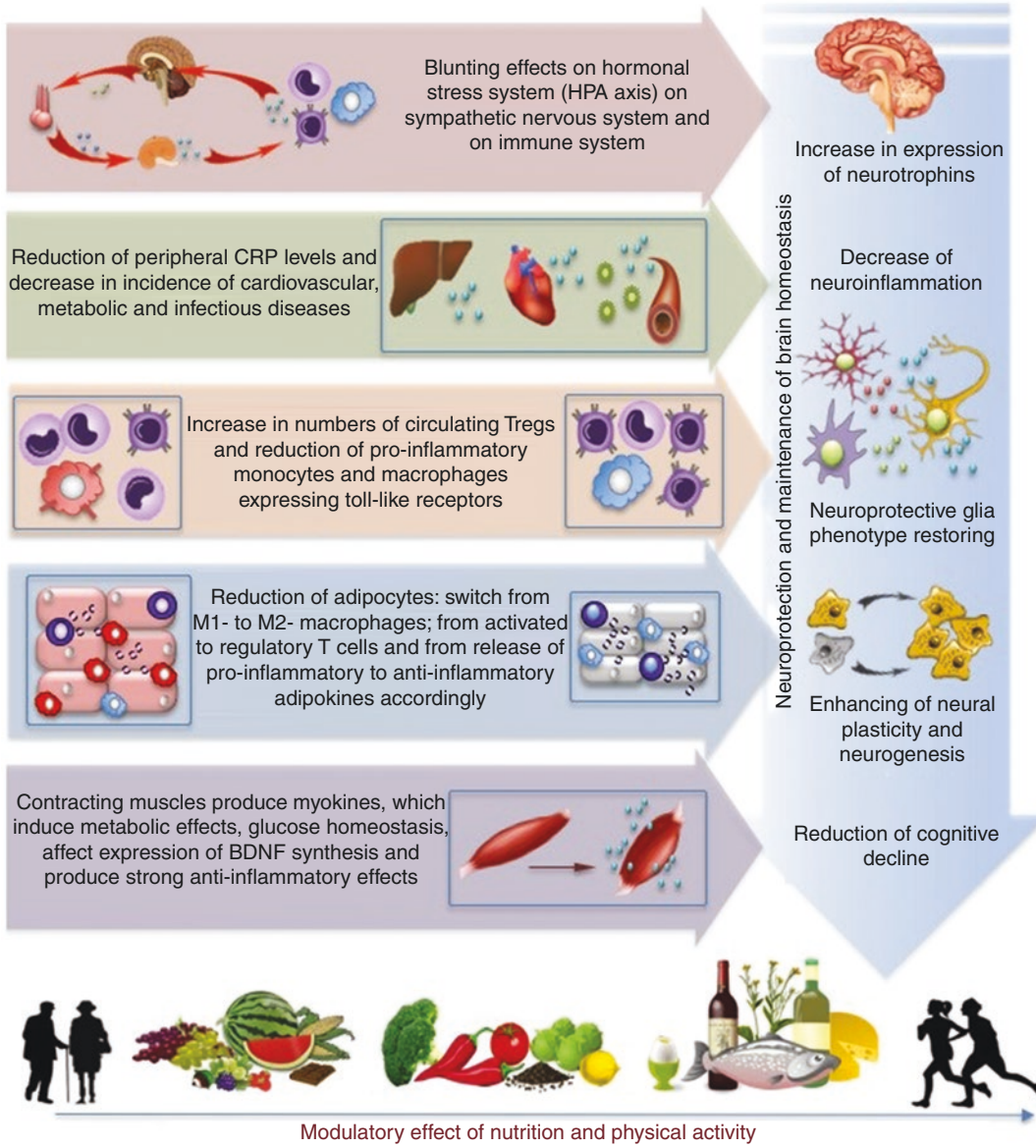


Fig. 14.4 Potential effects of nutrition and physical activity on immunoneurosenescence. HPA, hypothalamic-pituitary-adrenal axis. (Modified from Di Benedetto et al. [65]. https://doi.org/10.1016/j.neubiorev.2017.01.044)

Nevertheless, there are still many open questions concerning CR, which has been shown to be effective in improving the immune response in unchallenged animals, although it might compromise the host's defense against pathogenic infection and result in higher morbidity and mortality outside the laboratory. Moreover, CR has been shown to delay immunosenescence in animals, but this effect needs to be verified in humans. Furthermore, short-term CR may well be feasible, whereas long-term dietary restriction might have detrimental psychological and other effects in humans, especially in the older population, making its practicality questionable [2, 89, 90]. Nevertheless, the recent results from a multicentered, randomized clinical trial have

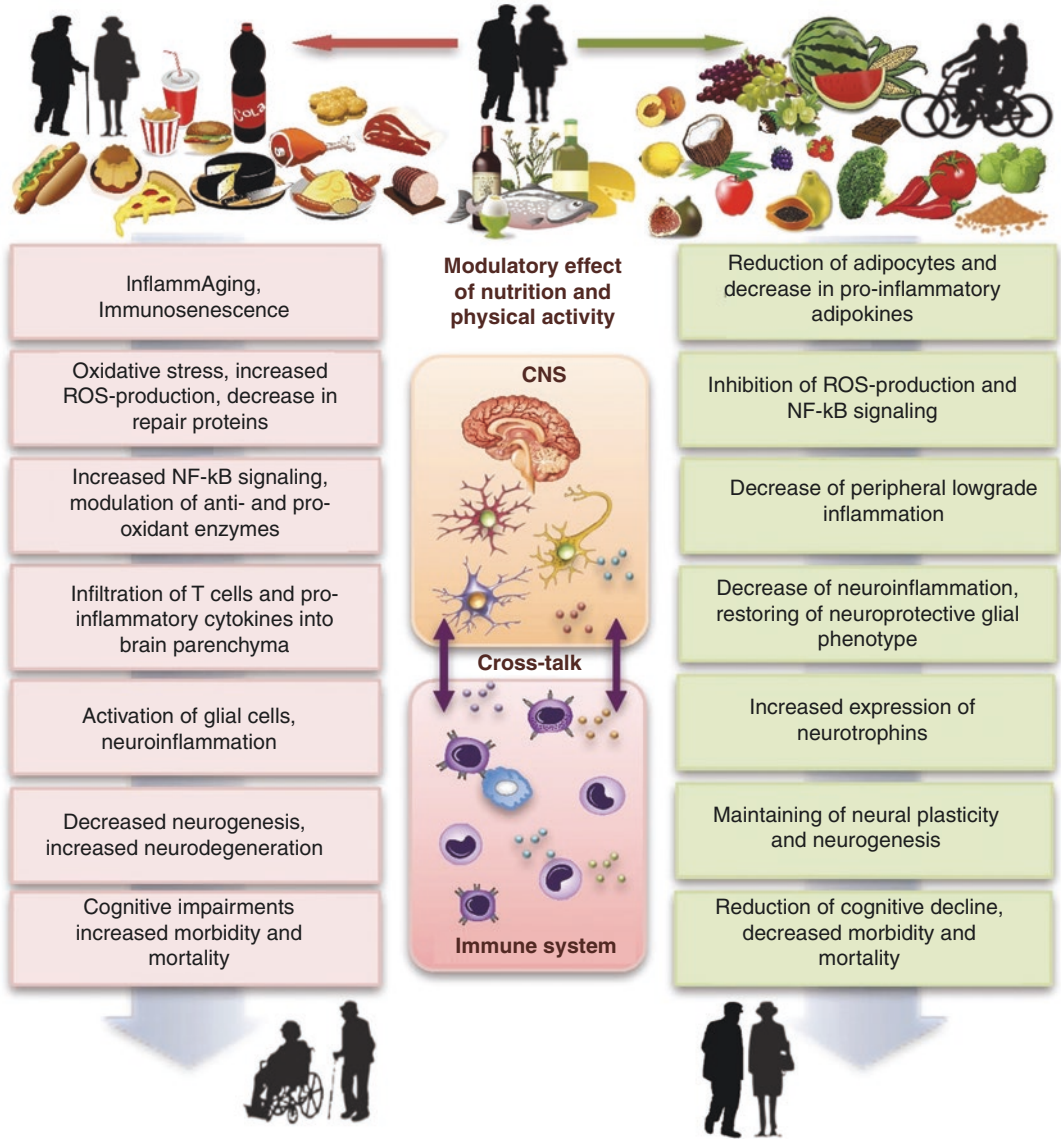


Fig. 14.5 An overview of the age-related effects and the modulatory potential of nutrition and physical activity on immunoneurosenescence. (Modified from Di Benedetto et al. [65]. https://doi.org/10.1016/j.neubiorev.2017.01.044)

demonstrated that long-term moderate CR without malnutrition induces a significant and persistent inhibition of inflammation in young and middle-aged healthy nonobese adults without impairing the cell-mediated immunity [91].

The optimization of nutrition may represent one of the first and theoretically easiest and cheapest strategies that can be employed to preserve health during aging [2]. The development of "elderly specific," functional foods, containing probiotics and/or prebiotics, may help in preventing the age-related disruption of the gut environment [92]. In fact, it has been demonstrated that the maintenance of a "healthy" gut microbiota during aging could help to delay or prevent the inflammaging process [93].

Immunostimulatory properties, such as the modulation of cytokine production or adjuvant effects on T-lymphocytes and NK activity, have been demonstrated for various health-promot-

ing *Lactobacillus* and *Bifidobacterium* strains [94–96]. It has been shown that 3- and 6-week interventions in elderly people can have positive effects, such as measurable increases of NK cells, tumoricidal activity, and monocyte phagocytic capacity [97]. A probiotic yogurt supplementation tested on elderly persons affected intestinal bacterial overgrowth. This intervention was able to normalize the response to endotoxin and modulate inflammatory markers in blood [98] as well as decrease the incidence of infections in this group of elderly people. Furthermore, a significant increase of phagocytosis, NK-cell activity, and production of IL-10 was also demonstrated following the probiotic supplementation in the elderly as well as the reduced production of the pro-inflammatory cytokines IL-6, IL-1β, and TNF [92, 99]. Boge et al. showed in two clinical trials that the consumption of a probiotic drink (containing *L. casei*) by elderly subjects for several weeks before and after an influenza vaccination led to a significant increase in the influenza-specific antibody titer, demonstrating the potential of probiotics in improving the protective efficacy of vaccination in the elderly population, in which it is usually considerably reduced [100, 101].

A seminal advance has been the realization that probiotic supplementation is likely to require "tailor-made" mixtures of different bacteria in order to achieve the desired effect. Atarashi et al. identified 17 strains of bacteria preferentially inducing colonic Tregs which reduced symptoms in models of colitis and allergic diarrhea in mice [102]. Thus, supplementation with tailor-made mixtures of probiotics may be effective in modulating immune responses where the use of single strains is ineffective. This may be one reason why the results of the many human probiotics supplementation trials have often been equivocal since mostly a single strain was used even if it was the "right" strain [2]. The mechanism of action of the mixed bacterial strains is the induction of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs by bacterial antigens, which in turn stimulate the intestinal epithelial cells to produce TGF-ß1, but not inflammatory IL-6, and TNF. Fecal samples from patients with inflammatory bowel disease and atopy have been reported to contain relatively low amounts of some of the same bacterial strains, suggesting their relevance to human disease as well as in the animal model.

In the context of supporting the "right" balance of gut bacteria, reversing our nutritional habits "back to mother nature" might represent an additional solution for the modulation of age-related inflammatory status and associated chronic and neurodegenerative diseases [2]. The accumulating data from in vitro, animal, and clinical studies provide evidence that a greater consumption of fruits, cereals, vegetables, legumes, and spices is associated with a lower risk of many diseases [103–108]. Consumption of diets consisting of natural foods can also raise the amounts of plant-based nutraceuticals in the body, such as antioxidants and anti-inflammatory agents. These nutraceuticals are known to cope with free radicals and to modulate the inflammatory signaling pathways in cells, suppressing the onset of age-related chronic conditions [2, 107, 109–111].

Several studies demonstrate that dietary polyphenols are able to repress inflammation by the inhibition of nuclear factor kappa-light-chain enhancer of activated B-cell (NF-κB) signaling [112–116]. Remarkably, plant extracts from strawberry, mulberry, and blueberry were shown to contain antioxidants that are able to activate the antioxidant defense system and to counter the deleterious effects of irradiation by reducing oxidative stress and inflammation [117]. A significant decrease in the levels of inflammatory cytokines was demonstrated by the administration of the diet supplements containing pomegranates, figs, or dates [118].

Nutritional intervention including micronutrients and vitamins has also been recognized as a practical, cost-effective approach to attenuate age-associated declines in immune function, vaccination efficiency, and resistance to infectious and neoplastic diseases. The importance of micronutrients, trace elements, and vitamins in proper immune functioning is clear, and deficiencies in one or more of these nutrients may impair practically all forms of immunity [2]. Among

them, zinc is an essential element of which the significance to health is indisputable, as discussed above [119]. It has been shown that zinc supplementation enhances the innate immune responses by increasing phagocytosis and T-cell functionality [120]. An increase in serum zinc concentration was associated with an increase in the number of T cells [119]. Zinc supplementation was also able to improve the generation of NK cells from $CD34^+$ cell progenitors through the increased expression of the GATA-3 transcription factor [7].

Vitamin C has also been found to positively modulate immunosenescence and inflammaging, representing two main hallmarks of human aging. Moreover, it has been shown to epigenetically regulate genome integrity and stability, indicating a key role of vitamin C in healthy aging [121].

Vitamin E supplementation has been demonstrated to improve immune responsiveness in healthy elderly individuals by the enhancement of cell-mediated immunity [122]. It was also able to reverse many of the T-cell age-associated defects, including reduced levels of phosphorylation of critical signaling proteins, as well as to improve defective immune synapse formation. Vitamin E also enhances the production of IL-2, the expression of several cell cycle control proteins, and T-cell proliferation [123]. Intake of vitamin E above recommended levels has been shown to enhance T-cell function in aged animals and humans. This effect is believed to contribute to an improved resistance to influenza infection and to a reduced incidence of upper respiratory infections in elderly people [1, 124].

Here, we can provide only a few examples, limited to spices and fruits and their bioactive components, known to modulate different stages of tumorigenesis, including tumor cell survival, proliferation, invasion, and angiogenesis. The anticancer activities of spices are mediated primarily through the suppression of inflammation. Bioactive components of spices, such as eugenol and 6-gingerol, prevent the release of TNF and IL-1β [125] in vitro stimulated macrophages [126]. Curcumin has been shown to suppress the inflammatory mediators NF-κβ and COX-2 [127], anethole inhibits NF-κβ activation and cytokine production [128], and cinnamaldehyde blocks the age-related activation of NF-κβ and targets inflammatory COX-2 as well as induces nitric oxide synthase (NOS) [129]. Ursolic acid, which is present in many fruits, including apples, pears, plums, bearberries, loquat, jamun, and rosemary, has been found to exert antitumor activity against colon cancer [130], breast cancer [131], nonsmall cell lung cancer [132], pancreatic cancer [105], melanoma [133], multiple myeloma [134], cervical cancer [135], and prostate cancer [108]. Also, several other nutraceuticals have been shown to exert antitumor and anti-inflammatory activities.

Since oxidative stress and inflammation are two of the major triggers of age-related pathologies and neurodegeneration, the consumption of phytochemicals from fruits, vegetables, herbs, and spices may exert relevant immunomodulatory and anti-inflammatory activities also in the context of the aging brain [86]. Compelling evidence has shown that dietary phytochemicals, particularly polyphenols, have properties that may suppress neuroinflammation and prevent toxic and degenerative effects in the brain. The mechanisms by which polyphenols exert their action are not fully understood, but it is clear that they have a direct effect through their antioxidant activities. They have also been shown to modulate intracellular signaling cascades, including the PI3K-Akt, MAPK, Nrf2, and MEK pathways. Polyphenols also interact with a range of neurotransmitters, illustrating that these compounds can promote their health benefits in the brain through a direct, indirect, or complex action [136]. Approaches with multiple antioxidant nutrients to block the oxidative stress related to the systemic and brain inflammation pathways may, therefore, prevent or delay the cognitive impairments by preventing microglia aging [137].

As a framework of balanced multicomponent diet strategy, adherence to a Mediterranean diet enriched with fruits, vegetables, nuts, and unsaturated fatty acids promotes neuroprotection by decreasing inflammation, restoring cerebral blood flow and volume, inhibiting neurodegeneration, and enhancing neural plasticity by increasing neurogenesis [138].

Flavonoids, plant polyphenolic compounds, which are abundant in fruits and vegetables, exhibit a wide variety of biological effects, including antioxidant, free-radical scavenging, and anti-inflammatory properties. Luteolin, a flavonoid found in high concentrations in celery and green pepper, has been shown to reduce the production of pro-inflammatory mediators and to inhibit the JNK and AP-1 signaling pathway in mice [139]. It was therefore suggested that luteolin may inhibit neuroinflammation and improve cognition in the otherwise healthy aged by constraining brain microglia [140].

Phenolic acids, an important class of polyphenols, are abundantly present in berries, nuts, coffee, tea, and whole grains. They exert their antioxidant and anti-inflammatory activities by the attenuation of microglial activation; the significant inhibition of the production of TNF, IL-6, IL-1β, and NO; and the reduction of mRNA and protein levels of COX-2 and iNOS [141]. Also, retinoids and carotenoids are known as potent antioxidants and anti-inflammatory agents having neuroprotective properties [142]. Sesamol, a phenolic lignan from sesame supplementation, prevents systemic inflammation-induced memory impairment [143].

Recently, much interest has focused on the suggested anti-inflammatory and neuroprotective effects of dietary-derived polyphenols and the long-chain n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), rendering these molecules as potential candidates for use in preventative and therapeutic strategies to reduce the risk of age-related chronic neurodegenerative diseases [144–146]. Both DHA and EPA can reduce neuroinflammation and cognitive decline. In particular, it has been demonstrated that EPA positively influences mood disorders and DHA maintains normal brain structure [147]. Supplementation with EPA may attenuate neuroinflammation by inhibiting microglial activation and microglia-produced pro-inflammatory cytokines and by enhancing the expression of astrocyte-produced neurotrophins and their receptors [148].

In general, many studies with animal models support the protective effects of n-3 PUFA supplementation under different conditions. However, studies to demonstrate clear benefits on human subjects toward a particular disease have not been conclusive, probably due to heterogeneous dietary habits and the population being in different demographic conditions. Since many botanical polyphenols can also up- and downregulate the same pathways, future studies may need to include testing for combined effects of DHA and these polyphenols [149]. Thus, more clinical studies are needed to determine the amounts of dietary agents needed to delay aging and age-related diseases and to investigate their effects on different age groups [107]. Furthermore, when identifying dietary approaches to promote healthy brain aging, a holistic approach should be considered, including nutrition, exercise, and lifestyle factors, which not only target the brain but also overall cardiovascular, immune, and metabolic health [144].

Several interventions, including different types of exercises, have been proposed to restore immune and neural functions in elderly people. Physical exercise and physical activity in later life might target modifiable risk factors of aging, such as low-grade inflammation, inducing immunoprotective and neuroprotective functions (Fig. 14.5) [150, 151]. Numerous studies have revealed the anti-inflammatory and antioxidative effects following physical activity (Figs. 14.4 and 14.5), which potentially exert beneficial effects on neuroplasticity, affect the expression of neurotrophins, and therefore help to maintain normal neuronal functions [152]. Barrientos and colleagues observed an inhibition of neuroinflammation (caused by infection) in rats performing exercises and an increased induction of BDNF mRNA in the brain of otherwise sedentary animals [153]. Further studies with aged mice revealed that wheel running inhibits the pro-inflammatory state of microglia and their ability to proliferate, thus inducing a pro-neurogenic phenotype [154].

Certainly, the anti-inflammatory effects of exercise are mediated through multidirectional pathways. Some of them are exerted on adipose tissue, skeletal muscles, immune system cells, and the cardiovascular system (Figs. 14.4

and 14.5). These effects include the modulation of anti-inflammatory and pro-inflammatory cytokine profiles, redox-sensitive transcription factors, and antioxidant and prooxidant enzymes and repair proteins [155]. Regular physical activity combined with nutritional interventions can reduce the size of adipocytes and attenuate inflammation in adipose tissue by phenotype switching from pro-inflammatory M1-type to anti-inflammatory M2-type macrophages (Fig. 14.4). In addition, a reduction in the numbers of circulating pro-inflammatory-type monocytes and an increase in the numbers of Tregs were found in the peripheral blood of individuals following physical activity [156, 157].

Recent evidence suggests that contracting muscles release myokines [2, 158–162], which affect the synthesis of BDNF in the dentate gyrus of the hippocampus [162]. Physical activity may have a buffering role on the hormonal stress-responsive systems (Fig. 14.4), such as the HPA axis and the sympathetic nervous system [163], thus maintaining immunoprotective and neuroprotective functions. It has been also shown that regular exercise is associated with an improved immune responsiveness to influenza vaccination in older adults. The exercise-related increase in the antibody titer, T-cell function, macrophage response, improvement of the Th1/Th2 cytokine balance, the level of pro-inflammatory cytokines, and changes in naive/memory cell ratio have also been reported [164].

Taken together, one might conclude that nutritional intervention in combination with physical activity activates and/or modulates the release of hormones, myokines, and cytokines as well as modulate the expression of various immune-reactive molecules, which all contribute to anti-inflammatory effects and the possible attenuation of immunosenescence and neuroinflammation.

Conclusions

Around the world, especially in many European countries, the older segment of the adult population is growing in size and proportion [165]. In dealing with this demographic change, what people make of the added years of their lives will be the most important aspect but will only be advantageous to the individual and to society at large if people can remain active participants in daily life and work [65]. Maintaining immunological and cognitive functions will be paramount, but their performances are known to decrease with increasing age, even in overtly healthy individuals. Preventing and/or attenuating immunosenescence and inflammation and delaying cognitive decline are probably the most effective measures for postponing the point of time at which individuals are no longer able to lead an independent life [65]. Effective pharmacological treatments, especially for cognitive but also for the immune decline, remain unavailable. Therefore, we will need a more holistic view of the aging process, where the dynamics of the interaction between environment, intestinal microbiota, and host must be taken into consideration. We should also take into account the age-associated physiological changes in the gastrointestinal tract as well as age-related modifications in lifestyle, nutritional behavior, and impaired functionality in the immune system of the elderly population [2, 65]. Some modifiable lifestyle factors, such as poor diet and physical and cognitive inactivity, have been identified as being associated with an increased risk of cognitive and immune decline [166]. Encouragingly, exercise can have a protective effect, even if initiated in advanced old age [2, 65, 167]. The intake of antioxidant nutrients reduces both systemic inflammation and neuroinflammation during aging [168]. Due to the fact that diet component targets are different, combining different nutrients acting on convergent anti-inflammatory pathways may result in an increased anti-inflammatory efficacy [144, 169, 170].

In this chapter, we have described the potential basic processes underlying age-related decline, namely, immunosenescence and a progressive increase in neuroinflammation characterized by an increased glial activation, elevated steady-state levels of inflammatory cytokines, and a decreased production of neurotrophic molecules as well as potential positive effects of nutrition and physical exercise. It is our

hypothesis, as partly summarized here, that a judicious combination of dietary interventions and exercise, cognitive training, and a pharmacological manipulation of immunosenescence and inflammatory processes will eventually deliver an optimal individualized regime for the maintenance of the best possible immune and cognitive functions over the lifespan of every individual.

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Vitamins and Allergic Asthma

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Key Points

- Treatment of asthma mainly relies on avoiding the triggers of asthma and use of a bronchodilator and anti-inflammatory medications.
- Evidence recently emerged suggesting that vitamins with antioxidant and anti-inflammatory properties, particularly vitamin C, D, and E, may modulate the inflammatory process of asthma.
- The current evidence is not conclusive to support the beneficial effect of vitamin C, D, and E supplementation in patients with asthma.

Introduction

Asthma is one of the most prevalent chronic respiratory diseases worldwide. In 2016, it was responsible for 0.77% of total deaths and 0.99% of total disability-adjusted life years (DALYs) globally [1]. According to the World Health Organization (WHO), it is estimated that more than 235 million people suffer from asthma [2]. It is estimated that additional 100 million people suffer from asthma by the year 2025 [3]. Asthma is a condition that likely results from complex interactions between genetic and multiple environmental factors [4]. Allergens, infections, air pollution, airway hyperreactivity, obesity, and tobacco smoke are some of the identified risk factors for developing the asthma [5]. The rapid increase in asthma incidence may be a consequence of environmental or lifestyle changes rather than genetic influences [6]. Exposure to the environmental factors may disrupt the balance of oxidant and antioxidant status in the body and may contribute to the development of asthma [7]. Therefore, maintaining this balance may have a role in the prevention or management of asthma [8].

The body has developed several antioxidant mechanisms to neutralize the effect of oxidants, including enzymatic as well as non-enzymatic mechanisms. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, and catalase, while non-enzymatic antioxidants include vitamin C, vitamin E, and glutathione [9]. The increase in oxidants can overwhelm the body endogenous antioxidant systems, leading to cellular injury and asthma manifestations [8]. Owing to this fact, researchers hypothesized that adding antioxidants may help to eliminate oxidants. As a result, the researchers' interest has been increased in the antioxidant system. Several observational studies reported that there is an inverse association between intake of antioxidants (such as vitamin C and vitamin E) and asthma. In addition, further researches unveiled the immunomodulatory role of vitamin D in the body. Vitamin D seems to battle airway hyperresponsiveness through different mechanisms compared to other vitamins. In order to confirm the association between the appropriate use of

antioxidants and vitamin D and control of asthma, several clinical trials have been conducted. Moreover, because lifestyle during pregnancy has a vital role in the development of the offspring, numerous studies performed to investigate the association of maternal status and/or supplementation of each vitamin with the incidence of wheezing in their offspring [10–12]. In this chapter, we summarize the current findings on the efficacy of the most studied antioxidant vitamins (vitamin C and vitamin E) and vitamin D in the prevention or treatment of asthma and/or wheezing.

Vitamin C

Vitamin C (ascorbic acid) is a water-soluble vitamin and a potent antioxidant. Vitamin C is part of the glutathione peroxidase pathway proposed to scavenge free radicals and neutralize their oxidative damage to the lipid membrane [13]. Several studies reported that there is an association between a high intake of vitamin C and control of asthma-related symptoms [14]. For instance, Nakamura and colleagues investigated the association of antioxidant vitamin intake with asthma in Japanese preschool children. They reported that high intake of vitamin C may be associated with a reduced prevalence of asthma [14].

Mechanism of Action

Several mechanisms have been proposed for the beneficial effect of vitamin C in the prevention of asthma or a reduction of its severity. Vitamin C protects the respiratory epithelium through its antioxidant activity. The existence of vitamin C in the lining fluids of the respiratory tract would reduce the oxidative damage of inhaled oxidants [15]. Another mechanism is related to the anti-inflammatory characteristic of vitamin C. For instance, it inhibits the production of pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) [16]. In addition, vitamin C has been shown to affect the arachidonic pathway [17] and produce immune-modulatory and antiviral functions [18].

Maternal Intake of Vitamin C and Incidence of Asthma in Offspring

Most of the cohort studies demonstrated that there was no association between childhood asthma or wheezing and vitamin C intake by pregnant mothers. For example, the Finnish Type 1 Diabetes Prediction and Prevention Nutrition Study, which is a population-based birth cohort study with 5-year follow-up, demonstrated that there was no association between maternal intake of vitamin C and the risk of asthma in the offspring at 5 years of age [19]. Furthermore, the Viva birth-cohort study, a longitudinal research study of women and children, revealed that maternal intake of vitamin C was not significantly associated with the risk of wheezing in children at 2 years of age [20]. On the other hand, West and colleagues reported that higher maternal intake of vitamin C was associated with a reduced risk of wheezing [21].

Vitamin C Supplementation in Patients with Asthma

Because of the antioxidant and anti-inflammatory characteristic of vitamin C, it was postulated that vitamin C supplement could alleviate asthma exacerbations. Therefore, several studies were conducted to investigate the beneficial effect of vitamin C supplementation in patients with asthma. However, most of the studies reported that vitamin C supplementation has no effect on asthma severity. For example, in a randomized controlled trial (RCT) study that was performed by Nadi and colleagues, 1 gram vitamin C supplement was given to adults with asthma. After 1 month, the lung function was assessed. There was no significant difference in the forced expiratory volume-one second (FEV1) among individuals who received vitamin C before and after the study. Similarly, no difference was observed in the control group [22]. Moreover, Fogarty and colleagues reported that after 16 weeks supplementation with vitamin C, there was no significant difference in FEV_1, peak expiratory flow rate (PEFR), asthma symptoms, and health-related quality of life among intervention and control

Table 15.1 Clinical trials of vitamin C supplementation in asthma treatment

Study	Design	Country	Setting	No of participants (intervention: control)	Vitamin C dose in intervention arm	Control	Results
Nadi et al. [22]	RCT	Iran	Adults	30: 30	1000 mg/day	Placebo	Vitamin C supplementation has no beneficial effect on pulmonary function tests including FEV1 and FEV1/FVC
Fogarty et al. [23]	RCT	UK	Adults	20: 20	1000 mg/day	1. 450 mg/day magnesium chelate 2. Placebo	Vitamin C supplements may have modest corticosteroid-sparing effects
Fogarty et al. [24]	RCT	UK	Adults	20: 20	1000 mg/day	1. 450 mg/day magnesium chelate 2. Placebo	After 16 weeks, no significant difference was observed in FEV1, PEFR, and asthma symptoms between vitamin C and placebo groups

RCT randomized controlled trials, *y* years, *GINA* Global Initiative for Asthma, *CACT* Childhood Asthma Control Test, *ACT* Asthma Control Test, *SGRQ* St. George's Respiratory Questionnaire, *FEV* forced expiratory volume, *SCIT* subcutaneous immunotherapy

groups [24]. The results of the previous studies demonstrated that vitamin C alone might not have a significant effect on asthma exacerbation. Table 15.1 summarizes the latest findings on vitamin C supplementation in asthma treatment.

Implications for Clinical Practice

Several studies have investigated the beneficial effect of vitamin C in patients with asthma. Yet, there is no conclusive and strong evidence suggesting a high intake of vitamin C for the prevention of asthma or a reduction of its severity.

Vitamin E

Vitamin E is a fat-soluble vitamin that because of its antioxidant activity has been used in the management of asthma. To date, the association of dietary intake of vitamin E and risk of asthma was assessed by several studies. The first Nutrition and Health Survey in Taiwan demonstrated no significant association between vitamin E intake and risk of asthma [25]. Similarly, the Sweden birth cohort of 4089 newborn infants (BAMSE) showed that alpha-tocopherol intake was not significantly associated with increased risk of asthma [26]. In contrast, Burns and colleagues investigated the association of vitamin E and risk of asthma in a cohort of 2112 twelfth-grade students in 13 communities in the United States and Canada. They demonstrated that asthma was more common among students who consumed vitamin E less than 5.2 mg/d compared with students who consumed vitamin E more than 5.2 mg/d [OR, 1.48; 95% CI, 1.03–2.13] [27]. Likewise, Nakamura and colleagues assessed the association of the dietary intake of vitamin E and risk of asthma in preschool children. They measured the vitamin E intake by a 3-day dietary recall and the values were stratified into tertile groups. They reported that compared with children who were in the lowest tertile of vitamin E intake, children who were in the highest tertile were less likely to have asthma [OR, 0.32; 95% CI, 0.12, 0.85] [28].

Mechanism of Action

The possible mechanisms for the beneficial effect of vitamin E or its isoforms in the prevention or management of asthma are as follows. One of the mechanisms is the anti-inflammatory action of vitamin E. For instance, it reduces the production and release of pro-inflammatory cytokines such as IL-4, IL-5, IL-13, and interferon (IFN) [29]. Another mechanism is related to the antioxidant activity of vitamin E that protects tissue and cells from free radical damage generated by oxidized fats [29]. Furthermore, it reduces the recruitment of neutrophils into the airway [30]. Modulation of the airway hyperresponsiveness is another possible role for vitamin E in the management of asthma [29]. Recent experiments have defined different roles for different isoforms of vitamin E in asthma. Alpha-tocopherol has been proven to be protective against asthma, whereas gamma-tocopherol is involved in the pathogenesis of asthma [31].

Maternal Intake of Vitamin E and Incidence of Asthma in Offspring

Interventional studies investigating the effect of vitamin E supplementation in patients with asthma are scarce. Despite this, several studies have attempted to determine the effect of maternal vitamin E intake on the development of asthma or wheezing in the offspring. However, the findings are inconsistent. For instance, Devereux and colleagues reported that maternal intake of vitamin E was negatively associated with asthma in 5-year-old children [32]. The finding was replicated by Allan and colleagues who measured the dietary intake of vitamin E using a food frequency questionnaire (FFQ) and the values were stratified into quintiles. After adjusting for potential confounders, they observed that children of mothers who had highest vitamin E intake had 37% lower risk of doctor-confirmed asthma at age 10 years, compared to children of mothers who had the lowest vitamin E intake [OR, 0.63%; 95% CI, 0.41–0.98] [33]. On the other hand, the Danish National Birth Cohort on 44,594 participants demonstrated that maternal intake of vitamin E during pregnancy was not significantly related to risk of asthma in their offspring at age 18 months [RR, 1.01; 95% CI, 0.91–1.12] and 7 years [RR, 1.06; 95% CI, 0.82–1.38] [34]. Similar to the Danish National Birth Cohort study, the findings of the Finnish Type 1 Diabetes Prediction and Prevention Nutrition Study revealed that there was no significant association between maternal intake of vitamin E and risk of asthma in children at age 5 years [19]. Most of the studies that evaluated the association between maternal vitamin E intake and risk of wheeze revealed that higher intake of vitamin E was associated with reduced risk of wheeze during childhood. Supporting this, a recent systematic review and meta-analysis revealed that maternal intake of vitamin E was related to lower odds of asthma [OR, 0.98; 95% CI, 0.96–0.99] and wheeze during childhood [OR, 0.65; 95% CI, 0.26–0.75] [35].

Vitamin E Supplementation in Patients with Asthma

Few interventional studies assessed the effect of vitamin E supplementation in patients with asthma and the results are conflicting. Ghaffari and colleagues conducted a double-blind RCT to evaluate the effect of vitamin E supplement in children with moderate asthma. During the study, children in the intervention group received fluticasone and 50 mg vitamin E per day and the control group received fluticasone plus placebo for 8 weeks. They demonstrated that 50 mg/day vitamin E supplementation led to improvement in FEV1 and FEV1/FVC ratio [36]. On the other hand, Pearson and colleagues in a double-blind RCT assessed the beneficial effect of vitamin E in adults with mild to moderate asthma. In this study, individuals in the intervention group received 500 mg vitamin E for 6 weeks. The primary outcome was change in bronchial responsiveness to methacholine. In addition, they compared change in resting FEV_1, FVC, mean morning peak flow, and serum immunoglobulin E (IgE) in the intervention and control group before and after the intervention. After completion of the study, there was no significant difference in the primary and secondary outcomes

between two groups. Therefore, Pearson et al. concluded that supplementation of vitamin E for 6 weeks has no effect on asthma control [37]. Therefore, based on the available evidence, it is not possible to suggest vitamin E supplement for the management of asthma.

Implications for Clinical Practice

Due to the conflicting results and differences in the study design, especially the duration of follow-up, it is not possible to conclude that there is a significant association between vitamin E intake and asthma or wheeze in children, even though this relationship is more strong for wheezing. Therefore, according to the current evidence, it is important that pregnant women consume vitamin E in the recommended level and not more than it.

Combination of Vitamin E and C

Given the role of vitamin C in the recycling of vitamin E, it was possible to hypothesize that the combination of vitamin C and vitamin E might enhance the antioxidant and anti-inflammatory functions of each one.

Vitamin C and Vitamin E Supplementation

Sienra-Monge and colleagues measured nasal inflammatory response to ozone exposure in children with asthma. Participants were given randomly a daily supplement of vitamin E (50 mg/day) and vitamin C (250 mg/day) or placebo. The nasal lavage levels of two pro-inflammatory cytokines, IL-6 and IL-8, uric acid, and total glutathione (GSx) were measured. After exposure to ozone, the nasal lavage level of IL-6 was increased significantly in the control group. After 12 weeks of the study, the levels of IL-6 and IL-8 were decreased in both groups. However, the level of IL-6 was exclusively different between intervention and control group [38]. There is a systematic review regarding the efficacy of the combination of vitamin C and vitamin E for asthma and exercise-induced bronchoconstriction. Due to the scarcity of evidence, it was not possible to draw a firm conclusion about the beneficial effect of the combination of vitamin supplements for asthma [39].

Maternal Vitamin E and Vitamin C Supplementation and Incidence of Asthma in Offspring

Greenough and colleagues assessed the effect of vitamin C and vitamin E supplement during pregnancy and risk of asthma in the offspring. Using the data of VIP trial (vitamin C and vitamin E in pregnant women at risk for pre-eclampsia), they demonstrated no difference in the development of asthma between infants of mothers who received 1000 mg vitamin C and 400 IU alpha-tocopherol daily in the second years of their life and infants of mothers who received placebo. In addition, it was reported that healthcare utilization and cost of care was higher in infants whose mothers received vitamin C and vitamin E supplementation than the remaining group [40]. Altogether, the evidence is lacking to recommend the combination of vitamin C and vitamin E more than the dietary reference intake (DRI). In addition, due to the unknown detrimental effects of vitamin supplementation during pregnancy, future studies should consider this issue.

More studies are needed to verify the hypothesis that the combination of vitamin C and vitamin E can be useful in the management of asthma. In addition, future studies are warranted to determine the effect of a combination of vitamin C and vitamin E relative to each individual vitamin. This issue is important because it could impose additional cost to the population.

Implications for Clinical Practice

There is no strong evidence to recommend vitamin C and E for the prevention or management of asthma.

Vitamin D

Vitamin D is a molecule of interest in asthma. A general reason is that most populations throughout the world have low levels of vitamin D, while vitamin D is known to contribute to the various functions of the body, especially the immune system. In particular, the repeated observations of the relationship between asthma and vitamin D and the efficacy of vitamin D supplement in asthmatic patients have made this field of study interesting for the clinicians and researchers.

Vitamin D deficiency has become a global issue with more than 1 billion affected individuals with insufficiency or deficiency of vitamin D [41]. National Health and Nutritional Examination Survey (NHANES) in the United States reported that mean serum 25-hydroxyvitamin D decreased from 30 ng/ml in 1988–1994 to 24 ng/ml in 2001–2004 [42]. Given the increased nutritional demands of pregnant and lactating women and children, they are at increased risk of vitamin D deficiency [43]. Some studies showed that children of mothers with vitamin D deficiency are considered at higher risk of aeroallergen sensitization, acute respiratory tract infections (RTI), wheezing, and asthma [44–47]. Vitamin D deficiency has been identified in 3.4% [48] to more than 50% [49] of children with asthma and some experts suggest vitamin D measurement for all patients [50]. Serum concentration of 25(OH)D (calcidiol) is typically measured to investigate vitamin D status as 1,25(OH)2D (calcitriol) has a short half-life compared to calcidiol (15 hours versus 15 days), and its serum concentration is under tight regulation of parathyroid hormone (PTH). Table 15.2 summarizes serum 25(OH)D cut points used to define vitamin D status [51].

Epidemiologic studies have linked vitamin D to asthma in several different ways. Of note, there have been reported factors such as westernized lifestyle, reduced physical activities with subsequently reduced sun exposure in urban areas, and obesity that would correlate with both low serum 25(OH)D and asthma [52]. From the molecular viewpoint, vitamin D receptors (VDR) are expressed in bronchial smooth muscle cells (SMC), goblet cells, airway epithelial cells, and immune cells residing in the respiratory tract [53]. Vitamin D exerts immunomodulatory effects by a reduction of airway SMC mass and suppression of bronchial tree remodeling [53–55]. Airway epithelial cells and lymphocytes express 1-α hydroxylase enzyme and are able to convert 25(OH)D to the active form of 1,25(OH)2D [56]. It underscores the active role of vitamin D pathway in airways.

Table 15.2 Vitamin D status category

Vitamin D status	Serum 25(OH)D	Serum 25(OH)D
Sufficient	40–80 ng/ml	100–200 nmol/L
Insufficient	20–40 ng/ml	50–100 nmol/L
Mild deficient	10–20 ng/ml	25–50 nmol/L
Moderate deficient	5–10 ng/ml	12.5–25 nmol/L
Severe deficient	<5 ng/ml	<12.5 nmol/L

Vitamin D conversion from nmol/L to ng/ml (nmol/L × 0.4 = ng/ml)

Dietary intake of vitamin D (egg yolk, oily fish, and fortified foods) constitutes only 10% source of vitamin D in the human body. The major source of vitamin D comes from skin production of pre-vitamin D from 7-dehydrocholesterol (7-DHC) following exposure to ultraviolet B (UVB) rays. In northern latitudes and winters, sunshine exposure is reduced with a subsequent decrease in intrinsic synthesis of vitamin D [57]. Interestingly, asthma exacerbations occur in a seasonal pattern with the highest hospital admissions in winter [57]. However, increased air pollution and acute RTIs are also implicated in higher rates of asthma exacerbations in winter. Interestingly, these are not against the hypothesis of vitamin D defense. Vitamin D can modulate inappropriate inflammatory responses triggered by air pollutants [58], and exposure to air pollution is associated with reduced circulatory and cord levels of 25(OH)D [59]. Moreover, vitamin D is linked to anti-viral and antibacterial activities of the innate immunity [60], and reduced serum 25(OH)D level is associated with higher acute RTIs and asthma exacerbations [61]. With respect to these findings, vitamin D deficiency seems to play a role in an

asthma scenario. Here, current advances regarding the role of vitamin D in asthma pathogenesis and possible therapeutic opportunities using vitamin D supplementation for patients with asthma have been reviewed.

Vitamin D Pathway and Asthma: Genetic Susceptibility

Vitamin D, whether adsorbed from food or synthesized in the skin, is inactive and requires further activations through hydroxylation at carbons 1 and 25 [62]. Enzymes of cytochrome P450 family (CYP) are involved in vitamin D activation in liver and kidneys [62]. Recently, it has been shown that lymphocytes and airway epithelial cells also express vitamin D-1α-hydroxylase (*CYP27B1*) and are able to produce 1,25(OH)2D [56]. This finding highlights the active contribution of vitamin D to airway function [56]. This is further supported by the presence of VDR in airway epithelial cells, SMCs, and lymphocytes [54, 53]. Figure 15.1 summarizes vitamin D activation pathway in the human body [62].

Polymorphisms in *CYP24A1* (encoding 24-hydroxylase, a deactivating enzyme) [63] and *CYP2R1* (encoding 25-hydroxylase, a hepatic enzyme which converts all pre-vitamin D to circulatory 25(OH)D) [63] are associated with asthma and atopy in genome-wide studies [63]. However, the results regarding vitamin D binding-protein (VDBP, also known as Gc globin) and VDR are inconsistent, and no genetic susceptibility has been found considering polymorphisms in the *CYP27B1* gene [63]. With respect to the VDR gene, there is only rs7975232 (Apal) polymorphism associated with asthma in Chinese [64] and Canadian populations [65]. These genetic variations also affect serum 25(OH)D and VDBP affinity to vitamin D. Study of Caucasian asthmatic children proposed that polymorphisms in the *VDBP* gene such as rs2282679 have the strongest association with serum 25(OH)D compared to other mediators involved in vitamin D activation pathway as observed in [66]. In addition, genetic variations might affect response to vitamin D supplementation and therefore are of potential importance in individualized-based medicine. Recently, Neyestani et al. showed a nutrigenomic response to vitamin D supplementation in diabetic patients and such a response might exist in patients with asthma [67].

Other interesting aspects of genes encoding mediators of vitamin D activation pathway are epistasis and epigenetics. Epistasis or genetic interaction denotes to the different expression of

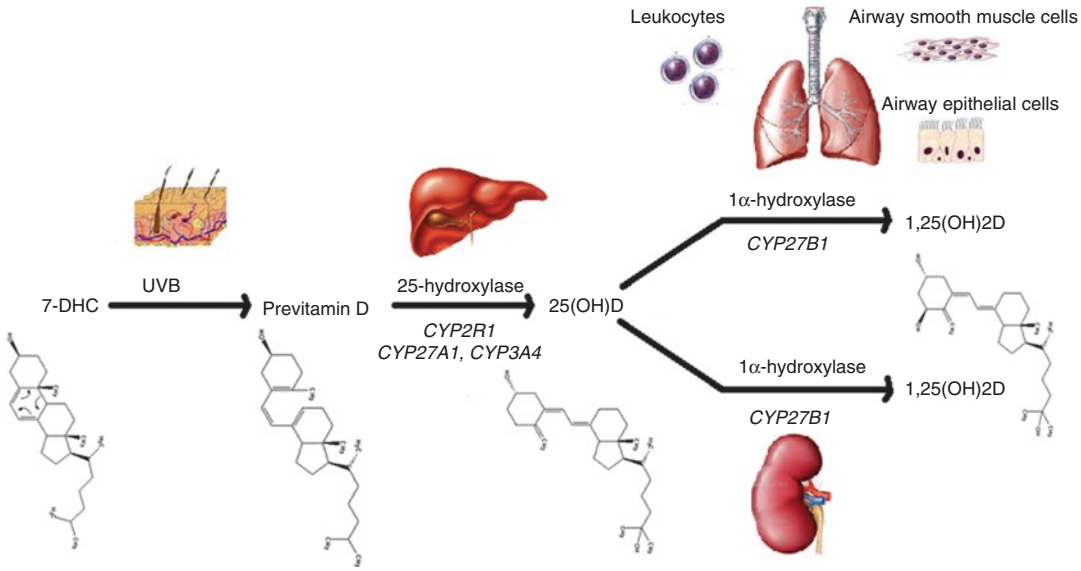

Fig. 15.1 Production of active vitamin D in the body and its receptors in the airway

one gene in the presence of a special variant of another gene. One of these gene-gene interactions was observed between *IL-10* and *VDR* genes in which the association of IL-10 variants with asthma susceptibility was dependent on *VDR* genotype [63]. Epigenetics or gene-environment interactions denote to changes in gene expression acquired during life without alteration in the genome sequence. Chromatin-modifying enzymes are important players in epigenetics. Recent studies have shown that multifunctional enhancers regulate VDR gene transcription and 1,25(OH)2D upregulates histone acetylation of *VDR* gene [68]. Vitamin D via VDR may affect expression of IL-10 as well as other pro-inflammatory cytokines by recruitment of deacetylase enzymes [69, 70]. It might also alter IL-10-VDR gene interactions related to an asthma scenario. Moreover, regulation of VDR expression by VDR analogs is different with respect to target cell environment as observed in osteoblasts and intestinal cells [71]. These interesting findings provide a genetic susceptibility to asthma with respect to vitamin D pathway, which can be modulated by other inherited genes and environmental factors.

Vitamin D Status and Asthma Incidence

Vitamin D has been proposed to play important roles in lung development during the fetal period [72]. During 14–32 weeks of gestation, concentrations of calbindin, a tissue 1,25(OH)2D-dependent calcium-binding protein, are increased in fetal lungs, and the addition of 1,25(OH)2D to the culture of lung fibroblasts would enhance pyruvate dehydrogenase activity and lactate production [73]. On the other hand, surfactant production by type II pneumocytes is induced by 1,25(OH)2D [74]. More than effects of vitamin D on lung development and maturation, prenatal vitamin exposure might modulate immune function in early life. Vitamin D supplementation in mothers enhances induction of tolerogenic immune responses early in life as observed with an increase in the expression of immunoglobulin-like transcripts in cord blood cells [75].

Maternal Serum 25(OH)D and Asthma Incidence in Offspring

It has been widely studied if maternal serum 25(OH)D correlates with risk of acute RTIs, wheezing, and asthma in the offspring. The results are inconclusive and even contradictory. In a cohort of 178 mother-child pairs with measured serum 25(OH)D during the third trimester of pregnancy, maternal serum 25(OH)D was not associated with cognitive function, child's growth, and cardiovascular system structure and function. On the other hand, children of mothers within the highest interquartile range for 25(OH)D (>30 ng/ml) were at the increased risk of acute RTIs and asthma by age of 9 years when compared to their peers in the bottom quartile (<12 ng/ml) [76]. In contrast to these findings, almost all studies in the same or different geographical areas reported a lower risk of asthma, atopy, wheeze, and acute RTIs in those within the top quartile for 25(OH)D compared to those within the bottom quartile. However, it should be noted that the risk ratio (RR) was always weak and mostly non-significant [77–80]. Differences in (a) the week of gestation at which measurements were done, (b) the factors which were applied to RR adjustment, and (c) the duration of follow-up might account for the discrepancies observed in the various studies. Furthermore, the documentation of asthma in children younger than 6 years is harder than adults with respect to limitations in spirometry and identification of respiratory symptoms, and thereby the results regarding asthma are relatively uncertain as compared to more objective outcomes like wheezing [81, 82]. The evidence is conclusive that its high serum level of 25(OH)D affords protection against wheezing in young children [77–80]. In particular, studies on cord serum samples collected at labor revealed an inverse association between 25(OH)D < 30 ng/ml and risk of wheezing but not asthma [45]. Another study on cord showed a relationship between vitamin D level and asthma. Rothers et al. defined three groups of people by 25(OH)D serum levels: people with sufficient levels (more than 20 and less than 40 ng/ml) as reference group, people with low levels (below 20 ng/ml), and people with high levels (more than 40 ng/ml) [46]. Children with low or high 25(OH)D lev-

els demonstrated an increased allergic sensitization compared to the reference group. However, no association with the risk of asthma and allergic rhinitis was found [46]. It would include both individuals with sufficient and high levels of vitamin D in the top quartile for 25(OH)D. Hence, the inconclusive results regarding maternal or cord 25(OH)D and risk of asthma might also lie in the inappropriate comparison of people within the top quartile with those in the bottom quartile. Cord 25(OH)D is positively correlated with the maternal serum concentration of 25(OH)D and maternal dietary intake of vitamin D [75].

Maternal Intake of Vitamin D and Asthma Incidence in Offspring

Maternal intake of vitamin D during pregnancy has been found to negatively associate with risk of allergic rhinitis and asthma in the offspring [83, 84]. In two large cohorts from Scotland (Aberdeen Maternity Hospital project with 1212 mother-child pairs) and the United States (Viva project with 1194 mother-child pairs), maternal intake of vitamin D was 548 ± 167 IU/day and 128 (ranged from 99 to 170) IU/day, respectively. Both cohorts found protective effects of high maternal intake of vitamin D against wheezing in early childhood [83, 84]. In a study of 1669 Finnish pregnant women, mean daily intake of vitamin D was 6.5 ± 3.8 microgram and was found to be negatively associated with the risk of asthma and allergic rhinitis in the offspring by the age of 5 years [85]. The authors of the Osaka Maternal and Child Health Study recruited 763 Japanese mother-child pairs. The mean daily intake of vitamin D and calcium in the mothers were 6.2 micrograms and 542.3 milligrams, respectively. In this study, mothers in the top quartiles compared to those in the bottom quartiles of vitamin D (9.1 vs 3.5 micrograms/day) and calcium (714.4 vs 364.8 milligrams/day) intake showed lower risk of wheezing with RR of 0.69 (0.42–1.14) and 0.57 (0.32–0.99), respectively [86]. Similarly, the Lifeways study, a prospective birth cohort in Ireland, revealed that a higher maternal intake of vitamin D was associated with lower odds of asthma (OR 0.93 per micrograms/day, 95% CI 0.89 to 0.98) in the offspring at any time-point over the 10-year follow-up period [87].

Studies previously recommended the daily intake of 10 micrograms [85]. Recently the institute of medicine has however recommended a daily intake of 15 micrograms (600 IU) vitamin D for pregnant women [51]. Research confirming this is abundant. For example, the authors in the study [88] suggest that 4000 IU vitamin D supplementation per day for pregnant women is safe and more efficient in achieving sufficient serum levels of 25(OH)D (>20 ng/ml) than 400 IU and 2000 IU daily supplementation. After the establishment of current recommended dietary allowance (RDA) for pregnant women, RCTs frequently reported beneficial effects of vitamin D supplementation of 400 IU and 1600 IU in improvement of metabolic status, insulin resistance, and gestational diabetes mellitus [89, 90]. However, studies are lacking for the effects of maternal vitamin D supplementation on metabolic status, bone mineral density, autoimmune diseases, allergic diseases, and pulmonary function of the offspring.

Serum 25(OH)D in Young Children and Asthma Incidence

The results of studies regarding serum 25(OH)D in young age and risk of asthma are not consistent. The Finnish cohort showed a higher incidence of atopy and asthma by age 31 in individuals who received more than 200,000 IU/day calcitriol in infancy [91]. The Avon Longitudinal Study of Parents and Children (ALSPAC), a population-based birth cohort from South West England, including data from 3323 children showed that serum 25(OH)D at the age of 9 is not associated with the risk of developing wheezing/asthma by age 15 [92]. Another cohort study on 689 Australian children revealed that those with insufficient serum levels of 25(OH)D at 6 years of age were more likely to develop atopy, airway hyperresponsiveness, and asthma by age 14 [93]. This risk was more prominent in boys [93]. Similarly, the association of vitamin D with peak expiratory flow rates (PEFR) is gender specific. In a cohort of 596 men and 611 women in Netherland, men with insufficient levels of serum

25(OH)D (<30 ng/ml) had significantly lower PEFR compared to their peers. This association was not present in women. Functional synergy or cross-reaction between 1,25(OH)D and 17-β-estradiol through estrogen receptor-α might regulate expression of VDBP, VDR, and vitamin D-inactivating enzyme (encoded by *CYP24A1*) [93, 94]. The factor that might underlie non-significant association of serum 25(OH)D with lung function and risk of asthma in women include lower physical performance and muscle strength, shorter height, and smaller lung capacities in women than men [95].

Vitamin D, Immune System, and Respiratory Tract Infections

Asthma is a chronic inflammatory disease of airways associated with bronchospasm and airway hyperresponsiveness [96]. About two-thirds of children and a half of adults with asthma suffer from allergic asthma; thus, anti-allergic agents are a considerable component of asthma management [96, 97]. To date, specific allergen immunotherapy (SIT) is the only curative treatment for allergic asthma (also known as extrinsic asthma) [96]. Serum 25(OH)D measured during pregnancy [46] or in blood samples of patients with asthma [48] have been shown to negatively associate with total IgE and eosinophil count, which are indicators of allergy [48, 46]. Moreover, sufficient serum levels of 25(OH)D but not high or low values are associated with lower risk of aeroallergen sensitization and positive skin prick test, emphasizing the immunomodulatory effects of vitamin D [46]. Rhinovirus and respiratory syncytial virus (RSV) infections are two important triggers of asthma exacerbations [98]. Vitamin D exerts immunomodulatory effects leading to the suppression of markers of allergy and improvement of the innate immune function that, in turn, would prevent RTIs and asthma exacerbations.

Innate Immunity

Vitamin D affects innate immune responses through suppression of inflammation (mainly by inhibiting nuclear factor kappa B (NF-κB) [99] and enhancement of antimicrobial peptide production (like cathelicidins) [100]. NF-κB can engage in crosstalk cascades leading to the production of pro-inflammatory cytokines including pattern recognition receptors (PRRs). 1,25(OH)2D induces the expression of PRRs such as toll-like receptor (TLR)2, TLR4, and CD14, while inhibiting the activity of NF-κB and therefore regulating the expression of PRRs [99, 101, 102]. TLR2 recognizes lipopeptides and CD14/TLR4 complex can detect lipopolysaccharides conserved in cell wall of many invading pathogens [103]. Upon detection, the body begins to fight pathogens by eliciting immune responses. Tight regulation of immune responses is the key to keep the immune homeostasis. Otherwise, an inappropriate inflammatory response might be detrimental to the host [103]. 1,25(OH)2D is among factors contributing to the regulation of the expression of PRRs [102]. In addition, TLR activation would induce the expression of VDR and VDBP. Thus, a feedback loop might include both TLR and vitamin D pathway activation [60], implying the importance of vitamin D in the defense against invading pathogens. More precisely, 1,25(OH)2D initiates a cascade leading to the activation of vitamin D response elements (Fig. 15.2) and thereby induces the expression of cathelicidin antimicrobial peptides (*CAMP*) and defensins (*DEFB4*) [104]. Cathelicidins are antimicrobial peptides implicated in the defense against viral and bacterial RTIs. There is a direct relationship between serum content of cathelicidins and 25(OH)D, and vitamin D supplementation was able to increase cathelicidin concentrations in people with vitamin D insufficiency [105]. Moreover, it has been shown that 1,25(OH)2D protects airways from exaggerated inflammatory responses induced by RSV infection while helping to maintain antiviral activity of the immune system [106]. Vitamin D supplementation in patients with asthma might enhance their antimicrobial responses and protect against acute RTIs. Majak and colleagues were the first to show that daily supplementation with 500 IU vitamin D in children with asthma reduces asthma exacerbations mediated by acute RTIs [107]. A meta-analysis recently confirmed the

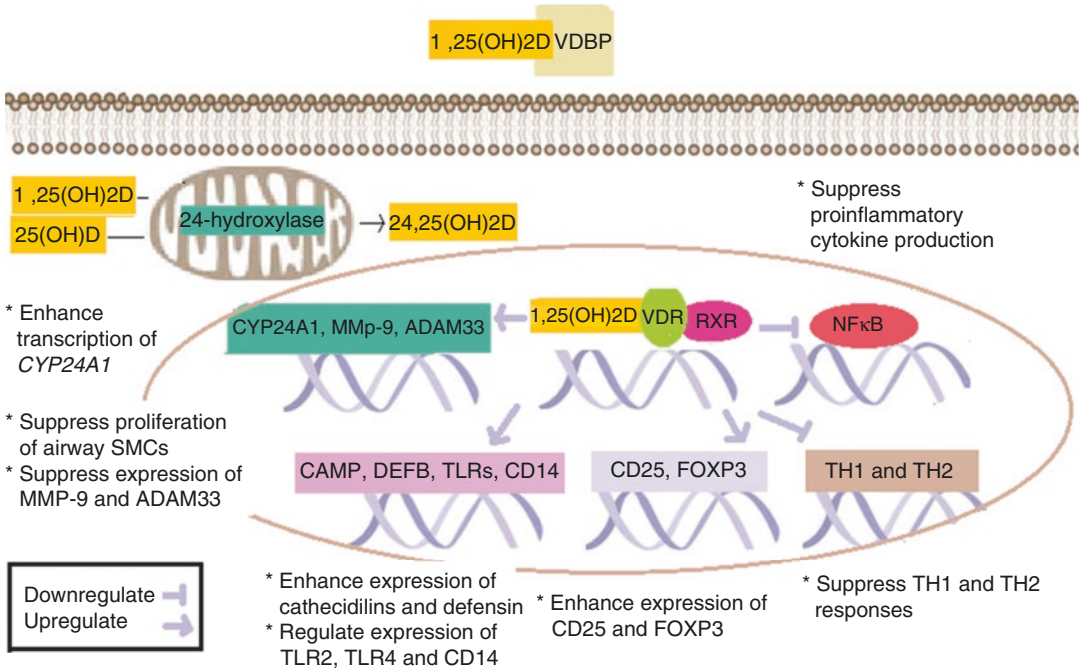

Fig. 15.2 Mechanism of action of the vitamin D in control of asthma

protective effect of vitamin D supplementation against acute RTIs (OR, 0.64; 95%CI, 0.49–0.84) [61]. However, there was a significant heterogeneity among included studies ($I^2 > 50\%$, $p < 0.001$) and the results might be influenced by publication bias [61]. In particular, the studies included populations other than of asthmatic or vitamin D insufficient subjects, for example, healthy subjects with sufficient levels of vitamin D. To draw more reliable results, an individual patient data meta-analysis has been launched.

Adaptive Immunity

The number of regulatory T (Treg) cells expressing CD25 and forkhead box P3 (FOXP3) is positively correlated with serum 25(OH)D in patients with asthma [108] ($r = 0.368$; $P = 0.021$). Interestingly, children with higher serum 25(OH)D (>30 ng/ml) demonstrate a better response to SIT in terms of symptom and medication scores, induction of regulatory responses, and corticosteroid needs [109]. A recent trial on 50 children with asthma found that 650 IU/day supplementation with vitamin D enhanced the expression of FOXP3 following injection immunotherapy in house dust mite-sensitized asthmatic children [110]. However, the production of IL-10 and transforming growth factor beta (TGF-β), two major cytokines of Treg family, remained unchanged [110]. Vassallo and Camargo were the first who addressed the question of how the ingestion of vitamin D would promote tolerogenic responses [111]. They proposed that early life vitamin D deficiency in concert with other factors constructs a pro-sensitization immune system [111]. Here, we extend this hypothesis to treat allergic asthma by proposing that vitamin D supplementation via oral route might modulate gut-associated lymphoid tissue function to promote tolerogenic responses in SIT. Such immunomodulatory effects of vitamin D are augmented when both allergens and vitamin D are introduced to the immune system via the same route. It is noteworthy that gut-associated lymphoid tissue is rich in antigen-presenting cells (APCs), which lead to the activation of the vitamin D pathway. Vitamin D affects the maturation of APCs and thereby modulates subsequent immune

responses [112]. Future studies investigating the potential effect of vitamin D supplementation with sublingual immunotherapy or oral immunotherapy are awaited.

As for Treg cells, helper T (Th)-1 and 2 cells would be influenced by vitamin D, though in a more complex way. Mast cells as one of the main effectors of Th2 responses are increased in bronchoalveolar lavage of patients with asthma [113]. The active form of vitamin D, 1,25(OH)2D, promotes apoptosis of mast cells and inhibits their maturation [114]. In addition, 1,25(OH)2D inhibits the production of Th2-type cytokines including IL-4 and IL-13 and Th2 differentiation from naïve cord T cells [115]. It is consistent with results of birth cohorts of maternal vitamin D status and aeroallergen sensitization in offspring. Treatment of both adult peripheral blood mononuclear cells (PBMC) [116] and naïve T cells derived from cord blood cells [115] with 1,25(OH)2D led to the inhibition of Th1 responses, including interferon gamma (IFN-γ) production. Th1/Th2 ratio is positively correlated with serum 25(OH)D in patients with asthma [108] ($r = 0.698$; $P = 0.0001$). Regarding the Th17 family, another subset of Th cells, vitamin D inhibits IL-17 production and thereby reduces chronic inflammatory responses in the airways [117].

Vitamin D and Lung Function and Structure

Lung Function

Studies of both healthy [118] and disease-affected subjects [119] confirmed the association between serum 25(OH)D with lung function. In the third National Health and Nutrition Examination Survey (NHANES), individuals within the highest quartile of serum 25(OH)D (>85.7 nmol/L) showed 126 ml and 172 ml increase in the forced expiratory volume in second 1 (FEV1) and forced vital capacity (FVC) volume compared to those in the lowest quartile of serum 25(OH)D (<40.4 nmol/L) [118]. Also, studies of patients with including chronic obstructive pulmonary disease (COPD) [120], cystic fibrosis (CF) [121, 122], and asthma observed that serum 25(OH)D correlates with lung function [119]. The cystic fibrosis foundation determined serum 25(OH)D level of 30 ng/ml as the cutoff for the optimal functioning of vitamin D in patients with CF [121]. However, other researched believed that the cutoff should be increased to 35 ng/ml [122]. Studies in children [123, 119] and adults [124] with asthma showed that serum 25(OH)D is positively associated with FEV1, FVC, and FEV1/FVC ratio. A study of children with asthma living in the United Kingdom confirmed that 25(OH)D was positively correlated with FEV1 ($r = 0.4$, $P < 0.001$) and FVC ($r = 0.3$, $P = 0.002$) [125] and that children with severe asthma had lower 25(OH)D levels compared to those with mild asthma (11.2 ng/mL versus 17 ng/mL) [125]. In another study of 435 Chinese adults with asthma, serum 25(OH)D was positively correlated with FEV1 ($r = 0.12$, $P = 0.02$) [124]. More interestingly, lower serum 25(OH)D was found to be associated with increased exercise-induced bronchospasm in Italian children with asthma [119].

On the other hand, there are studies that found not such an association between lung function and serum 25(OH)D. A study of 616 Costa Rican children with asthma showed that serum 25(OH)D was negatively associated with FEV1 and positively with airway hyperresponsiveness (PD20 < 8.58 mmol) which did not remain significant after adjustment for age, sex, body mass index (BMI), and parental education [48]. These results were replicated in children with asthma living in Thailand (latitude of 13° N) [126] and in adults with asthma living in Costa Rica (10° N) [127]. These areas are closer to the equator compared to the United Kingdom (53.5° N), China (35° N), and Italy (43° N). Lower sunshine exposure and subsequent lower vitamin D synthesis might be responsible for the difference in studies across geographical areas. Vitamin D deficiency (serum 25(OH)D below 20 ng/ml) was present in 3.4% of Costa Rican children [48], 29.7% of Costa Rican adults [127], 19.2% of Thai children [126], 88.9% of Chinese adults [124], and 51.1% of Italian children [119] with significant difference between low and high latitude areas

($p < 0.05$). It seems that in areas with high latitudes and higher prevalence of vitamin D deficiency, serum 25(OH)D is more strongly associated with lung function. It is thus possible to suggest that vitamin D sufficiency might not result in linear improvement in lung function. In addition, correlations between serum 25(OH)D and lung function had been always weak ($r < 0.4$) [125, 124].

Airway Remodeling

Vitamin D pathway modulates the function and proliferation of bronchial SMCs [53]. Microarray studies have demonstrated that 1,25(OH)2D alters the expression of genes involved in cell proliferation, growth, and survival [53]. Addition of 1,25(OH)2D to the airway SMCs inhibits platelet-derived growth factor-induced proliferation of SMCs [128]. It prevents from an increase of airway SMC mass. However, it does not increase apoptosis of proliferated SMCs [128]. Hence, early vitamin D supplementation might prevent airway remodeling in patients with asthma. Of note that such an effect has not been observed for corticosteroids [128]. Consistent with these findings, Gupta et al. observed that serum 25(OH)D was inversely associated with inhaled corticosteroid use and asthma exacerbations. They also found that airway SMC mass but not reticular basement thickness or epithelial shedding was inversely associated with 25(OH)D ($r = -0.6$, $p = 0.008$) [125]. On the other hand, vitamin D inhibits the expression of structural proteins implicated in airway remodeling [53]. Song et al. found that pre-treatment of airway SMCs with 1,25(OH)2D reduces the expression of matrix metalloproteinase-9 (MMP-9), disintegrin, and metalloproteinase domain-containing protein 33 (ADAM33) following exposure to serum of patients with asthma [129]. MMP-9 is involved in the breakdown of extracellular matrices and ADAM33 is a membrane-anchored protein involved in cell-cell and cell-matrix interactions [129]. These findings highlight that vitamin D might prevent airway narrowing and remodeling in patients with asthma.

Vitamin D Supplementation in the Management of Asthma

As above explained, vitamin D would exert anti-asthmatic effects in different ways. Vitamin D insufficiency is also associated with increased hospitalization due to asthma exacerbations in both children [48, 130] and adults [127]. Vitamin D insufficiency is more common among patients with severe asthma compared to patients with mild asthma and healthy individuals [127]. In addition, serum 25(OH)D is inversely correlated with airway SMC mass [125]. Hence, it seems reasonable to expect that vitamin D supplementation in patients with asthma might improve lung function and innate immune responses and alleviate allergic reactions and airway remodeling.

The results are conflicting regarding the beneficial effect of vitamin D supplementation in patients with asthma. Tachimoto and colleagues conducted a RCT investigating the efficacy of low-dose, short-term vitamin D supplementation in Japanese schoolchildren with asthma. Children aged 6–15 years were randomized to receive 800 IU/day vitamin D or placebo. After 2 months, changes in asthma control levels defined by Global Initiative for Asthma (GINA) were assessed. At the study endpoint, patients who received vitamin D has significantly better asthma control compared to those in the placebo group. In addition, as a secondary outcome of the study, childhood asthma control test scores were significantly improved in the vitamin D group compared with the placebo group [131]. Similarly, Yadav and colleagues assessed the therapeutic role of vitamin D in Indian children with moderate to severe bronchial asthma. Fifty children received 60,000 IU vitamin D per month and 50 children received placebo. They reported that a 6-month supplementation with vitamin D could reduce number of asthma exacerbations. Furthermore, vitamin D supplementation significantly decreased the need for steroids and emergency visits [132]. In contrast, several studies reported that vitamin D supplementation had no

beneficial effect on the management of asthma. For instance, Kerley and colleagues conducted a RCT to assess the effect of 15-week treatment with vitamin D in Irish children with asthma. Children aged 6 to 16 years were randomly allocated to receive either daily 2000 IU vitamin D or placebo. After 15 weeks, they observed no significant difference between vitamin D and placebo groups regarding in terms of asthma control [133]. Furthermore, two RCTs with large sample sizes that were conducted in adults with asthma revealed similar results. In the ViDiAs study, which was carried out in the United Kingdom, adults with asthma received a bolus dose of vitamin D. Participants were randomly assigned to receive 120,000 IU vitamin D or placebo each 2 months for a period of 1 year. The study showed that bolus-dose vitamin D supplementation did not influence time to exacerbation and the risk of upper respiratory infections [134]. The VIDA (Vitamin D Add-on Therapy Enhances Corticosteroid Responsiveness in Asthma) RCT reported the same results. In the VIDA study, 201 and 207 participants aged ≥18 years, respectively, received vitamin D supplementation and placebo. Participants in the intervention group initially received 100,000 IU vitamin D and then received 4000 IU per day for 28 weeks. They reported that vitamin D3 had no significant effect on first treatment failure or exacerbation in adults with persistent asthma and vitamin D insufficiency compared to placebo group [135]. Table 15.3 summarized the findings from clinical studies investigating the efficacy of vitamin D supplementation in patients with asthma.

Vitamin D supplementation may enhance response to corticosteroids which in turn reduces medication needs of patients with asthma. Cytochrome P450 family of enzymes are important players in the metabolism of not only vitamin D but also steroid drugs which are commonly used in moderate to severe asthma [136]. In a cohort of 734 children with asthma, harboring the CYP3A∗22 allele seemed to be associated with improved asthma control [137]. Of note, the CYP3A∗22 allele decreases the expression of hepatic CYP3A4 [137]. The activity of CYP3A family might justify why about 30% of patients with asthma are insensitive or resistant to glucocorticoid therapy. Interestingly, steroid-resistant asthmatics have lower serum levels of 25-(OH) D [123]. Furthermore, the addition of 1,25(OH)2D to PBMCs derived from patients with asthma-enhanced responsiveness to glucocorticoids and increased the production of IL-10. This observation was explained by inhibition of downregulation of glucocorticoid receptors following the addition of 1,25(OH)2D [70]. In another experiment, treatment of human airway SMCs with an active form of vitamin D altered chemokine expression and inhibited steroid resistance. Accordingly, vitamin D could be of benefit for patients with steroid-resistant asthma [138]. Serum 25(OH)D is inversely associated with inhaled corticosteroid use in children with asthma [123, 109]. Consistently, those patients with insufficient serum 25(OH)D are more likely to use more inhaled or systemic corticosteroids and have lower bone marrow density [139]. Future studies should be conducted to investigate skeletal benefits of vitamin D supplementation particularly in growing children and women who use corticosteroids. To date, there exist few clinical trials regarding vitamin D supplementation as an adjuvant therapy for patients with asthma. Vitamin D supplementation combined with immunotherapy reduced corticosteroid need in children with atopic asthma [110]. In addition, vitamin D supplementation averted RTIs and thereby reduced asthma exacerbations [107].

Implications for Clinical Practice

The current evidence is not strong enough to support the beneficial effect of vitamin D supplementation in patients with asthma. Large-scale and well-designed trials are essential to better understand the effectiveness of vitamin D in people with asthma.

Table 15.3 Clinical trials of vitamin D supplementation in asthma treatment

Study	Design	Country	Setting	No of participants (intervention: control)	Vitamin D dose in intervention arm	Control	Results
Solidoro et al. (2017) [139]	QE	Italy	Adults	55	100,000 IU IM once, then 50,000 IU/week and 400 IU/day	No control	After 12 months, the number of asthma exacerbations and blood level of eosinophils decreased significantly. The median annual values of FEV1 were higher than in the year before supplementation
Jensen et al. (2016) [140]	RCT	Canada	1–5 y	11: 11	At baseline 100,000 IU During the study 400 IU/day	At baseline placebo During the study 400 IU/day vitamin D3	After 3 months, no significant difference in rate of acute-care visits and oral corticosteroids between intervention and control groups
Tachimoto et al. [131]	RCT	Japan	6–15 y	54: 35	800 IU/day	Placebo	After 2 months, there was a significant difference in asthma control by GINA and CACT scores between intervention and control groups
Kerley et al. [133]	RCT	Ireland	6–16 y	19: 25	2000 IU/day	Placebo	After 15 weeks, there was no significant difference in asthma control (GINA and CACT scores) between intervention and control groups
Martineau et al. [134]	RCT	UK	16–80 y	125: 125	120,000 IU/each 2 months	Placebo	After 12 months, no significant difference in asthma control (time to first severe exacerbation, ACT score, FEV1, SGRQ score) between intervention and control groups
De Groot et al. (2015) [141]	RCT	The Netherlands	≥18 y	22: 22	Single dose of 400,000 IU	Placebo	After 9 weeks, no significant difference in asthma control (ACQ and AQLQ scores) and blood or sputum levels of eosinophils and neutrophils between intervention and control groups
Castro et al. [135]	RCT	USA	≥18 y	201: 207	100,000 IU once, then 4000 IU/d for 28 weeks	Placebo	After 28 weeks, no significant difference in asthma exacerbation and treatment failure between intervention and control groups
Yadav et al. [132]	RCT	India	3–14 y	50: 50	60,000 IU/month	Placebo	After 6 months, vitamin D supplementation significantly reduced the number of exacerbations, the requirement of steroids, and the requirement of emergency visits and increased the peak expiratory flow rate in the intervention group, compared to placebo group. Control of asthma was achieved earlier in the intervention group.

Arshi et al. (2014) [142]	RCT	Iran	10–50 y	64: 66	100,000 IU IM once, then 50,000 IU/ week	Placebo	After 24 weeks, the FEV1 was significantly better in the intervention group than that in the control group
Baris et al. (2014) [143]	RCT	Turkey	5–15 y	1. SCIT +650 IU vit D (n = 17) 2. SCIT alone (n = 17) 3. Pharmacotherapy (n = 20)	SCIT +650 IU vitamin D	1. SCIT 2. Pharmacotherapy	After 12 months, total asthma symptom score, total symptom score, and total medication scores were significantly lower in the SCIT + vitamin D and SCIT alone groups than pharmacotherapy group
Lewis et al. (2012) [144]	RCT	USA	6–17 y	15: 15	1000 IU/day	Placebo	After 12 months, no significant difference in ACT score or FEV1 value between intervention and control groups
Majak et al. [107]	RCT	Poland	5–18 y	24: 24	Budesonide 800 mg daily and 500 IU daily	Budesonide 800 mg daily and placebo	After 6 months of treatment, there was a significant improvement in ATAQ score and FEV1 in both budesonide + vit D and budesonide groups. During 6 months of treatment, the number of children who experienced asthma exacerbation was significantly lower in the budesonide + vit D group than that in the budesonide group.
Urashima et al. (2010) [145]	RCT	Japan	6–15 y	217: 213	1200 IU/day	Placebo	After 4 months, there was a significant difference in occurrence of asthma attack in intervention and control groups
Majak et al. (2009) [146]	RCT	Poland	6–12 y	18: 18: 18	Prednisone 20 mg + 1000 IU/ week	1. Prednisone 20 mg 2. Prednisone 20 mg + placebo	After 12 months, the asthma symptoms score was decreased in all the three groups. There was no significant difference between the groups.
Schou et al. (2003) [147]	RCT	Denmark	Children	8: 9	600 IU/day	Placebo	After 4 weeks, no significant difference in peak expiratory flow rates, FEV1, and asthma symptom scores between intervention and control groups

QE quasi-experimental, *RCT* randomized controlled trials, *y* years, *IM* intramuscularly, *GINA* Global Initiative for Asthma, *CACT* Childhood Asthma Control Test, *ACT* Asthma Control Test, *SGRQ* St. George's Respiratory Questionnaire, *FEV* forced expiratory volume, *SCIT* subcutaneous immunotherapy

Conclusions

Theoretically and according to the mechanistic pathway, vitamin C, E, and D may have beneficial effect in the management of the asthma exacerbation. However, the findings of the performed studies to support these theories are inconclusive and inconsistent. Therefore, the clinical applicability warrants further well-designed RCTs with greater number of participants.

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Nutrition, Immunity, and Food Intolerances

16

Tracy Bush

Contents

Key Points

- The way that society views food intolerances may not be optimal at the current time. Scientific research has suggested that healthcare must be looked at from a new perspective.
- Physician education and training are overdue for an upgrade. Patient care is no longer as cut and dried as it has been, and teaching methods must reflect this as well.
- Evidence-based proof puts a limitation on how patients are diagnosed and treated. If a patient cannot be viewed as

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their own person within typical given perimeters, misdiagnosis may mask health symptoms, causing failure of optimal patient treatment.

- The effects of our foods, both positive and negative, should be viewed from the perspective of beginning from the inside out. Cause and effect is not researched according to where our bodies originate true symptoms but rather to what.
- Food elimination testing has no negative effects as long as adequate nutrition is continued while testing. Encouraging or recommending a continued diet of foods that may aggravate symptoms has multiple side effects.
- Recognizing that healing your immune system includes gut health is imperative. Without proper digestion, nutrients and adequate nutrition will not be absorbed properly by the body.
- Healthcare must include two key factors: (1) physicians who are professional enough to acknowledge when they must refer a patient to someone else that may have a better grasp on what the patient requires and (2) patients who must educate themselves to be an active part of their own healthcare plan which may include finding a physician that can wholly grasp their personal health needs.

Introduction

In a world where physicians are typically trained to treat the main symptom versus the core of the offending issue, patients are increasingly searching to dig deeper on the true cause of ailments alongside standardized treatment. The way the body reacts to different factors can sometimes make a correct diagnosis difficult. Patients are becoming more in touch with their bodies, including how to trace where the original ailments begin. With treatment alternatives expanding and medical professionals beginning to see the benefits of a broader range of whole health treatment plans for their patients, the medical world is being taken by storm. Foods are now one of the top sources of a patient's health examination whether seeming immediately relevant or not.

Subjects that have been overlooked in the past are now opening a door to patient healthcare and allowing for a spectrum of alternative treatments, when they were not considered in the past. That which was thought to be a symptom in one area of the body may actually be originating from a completely different part of the body. As expressed by Dr. Mark Hyman "That's because doctors are very well-trained to treat symptoms and diseases, but NOT to address the underlying imbalances that perpetuate illness" [1]. Treating the secondary area rather than the primary area leaves room for error and increases the treatment time for the patient. Increased treatment time then leads to possible unnecessary medications in addition to added stress from increased physician/patient time and medical costs as well as added side effects contributed to these factors. What has not been deeply delved into is where the stressors are originating from. Thankfully, the medical community is beginning to entertain the idea that immunoglobulin (Ig) E information plays a role in immune responses [2].

When our body reacts, it is trying to fix itself. This is true whether it involves a food, a scrape on the knee, or the common cold. Multiple systems work together to begin repairing itself but, in the process, may become overwhelmed when there is a glitch in the usual operation. Imagine that your body is an automobile—when you make sure it runs on the correct fuel and maintain it with the proper fluids and upkeep, everything moves along smoothly. When you try adding the wrong type of fuel or begin to slack on all the mandatory ingredients that are essential to keep the machine functioning optimally, things begin to buckle and break down. Your body does this as well—it can be repaired but there is no proof that complete repair can be achieved if too much damage has been done without prompt treatment. Based on the NCBI abstract *Nutrition and The Immune System: An Introduction*, "Nutrition is a critical determinant of immune responses and malnutrition is the most common cause of immunodeficiency worldwide" [3]. What if the majority of your hard-to-diagnose symptoms could be

decreased or improved altogether by changing the foods that you eat or avoiding certain foods altogether?

Allergy Versus Intolerance

To understand the connection of nutrition, immunity, and food intolerances, we must first have a clear understanding of the significant differences between a food allergy and a food intolerance. It is extremely important to recognize the variations to ensure the person being treated is kept as harm free as possible. With a food allergy, the immune system reacts to food. The body releases histamine to try to get rid of the offending food. The response that occurs with a true food allergy is known as anaphylaxis. "Anaphylaxis is a serious allergic reaction that comes on quickly and has the potential to become life-threatening" [4] (Fig. 16.1). Anaphylactic symptoms can present in one or several areas of the body (mouth, throat, lungs, gut, brain, eyes, nose, skin, circulation, or heart) and must be treated upon immediate exposure of the allergen. The directed treatment for anaphylaxis is epinephrine followed by immediate consultation of a physician or emergency medical response team member. Current studies estimate that 1 in 13 people has a food allergy.

Food intolerances typically do not cause anaphylaxis but can cause similar signs or symptoms of a food allergy. A majority of the body is affected in the gastrointestinal system which then circulates outward to other areas of the body but does not affect the immune system as in the case of anaphylaxis. Unlike a food allergy diagnosis, patients with food intolerances may go months or years undiagnosed due to the limited knowledge of how certain foods are adversely affecting their bodies. "Moreover, the diagnosis of food allergy (FA) may be problematic, given that nonallergic food reactions, such as food intolerance, are frequently confused with FAs" [5]. Because most food intolerance symptoms mimic other ailments, the correct treatment is often discovered in a time period beyond the initial symptom presentation and can cause prolonged symptoms which then turn into increased and additional symptoms. This too may add to the difficulty of establishing a correct diagnosis and proper treatment protocol. "Just because a food intolerance is not life-threatening does not mean it is not life-altering" [6].

One of the major hurdles of having a food intolerance is that many doctors and patients rely on evidence-based scientific proof. For centuries, science has been instilled with what explains cause and effect. Currently, there is another side of science that asks new questions to get new answers about our health. One example are the thoughts shared by Dr. Steven P. Novella "Good science is the best and only way to determine which treatments and products are truly safe and effective". That idea is already formalized in a movement known as evidence-based medicine (EBM). EBM is a vital and positive influence on the practice of medicine, but it has limitations and problems in practice: it often overemphasizes the value of evidence from clinical trials alone, with some unintended consequences, such as taxpayer dollars spent on "more research" of questionable value. The idea of science-based medicine (SBM) is not to compete with EBM, but a call to enhance it with a broader view: to answer the question "what works?", we must give more importance to our cumulative scientific knowledge from all relevant disciplines" [7]. So I ask the same question again—What if the greater part of your hard-to-diagnose symptoms could be decreased or improved altogether just by changing the foods that you eat or avoiding certain foods altogether? Unlike prescribing a medication, there are little to no side effects when a person changes their diet. While some practitioners are adamant on treatment that comes from a prescription pad, they could be just as insistent that they listen to how the patient's body is reacting to everything and to eliminate what does not work for each patient. Often, the reluctance in offering food elimination is the fear of malnutrition due to the vitamins, minerals, fats, and proteins that we have been told to eat for centuries. Although there are supplements and pills available to improve some of these issues, the only true treatment is through the use of whole foods [8]. For every food that can be eliminated, there is another to replace it. I will even go as far to say that discovering that you have a food intolerance may just make you healthier and open your eyes to the foods that you should have been

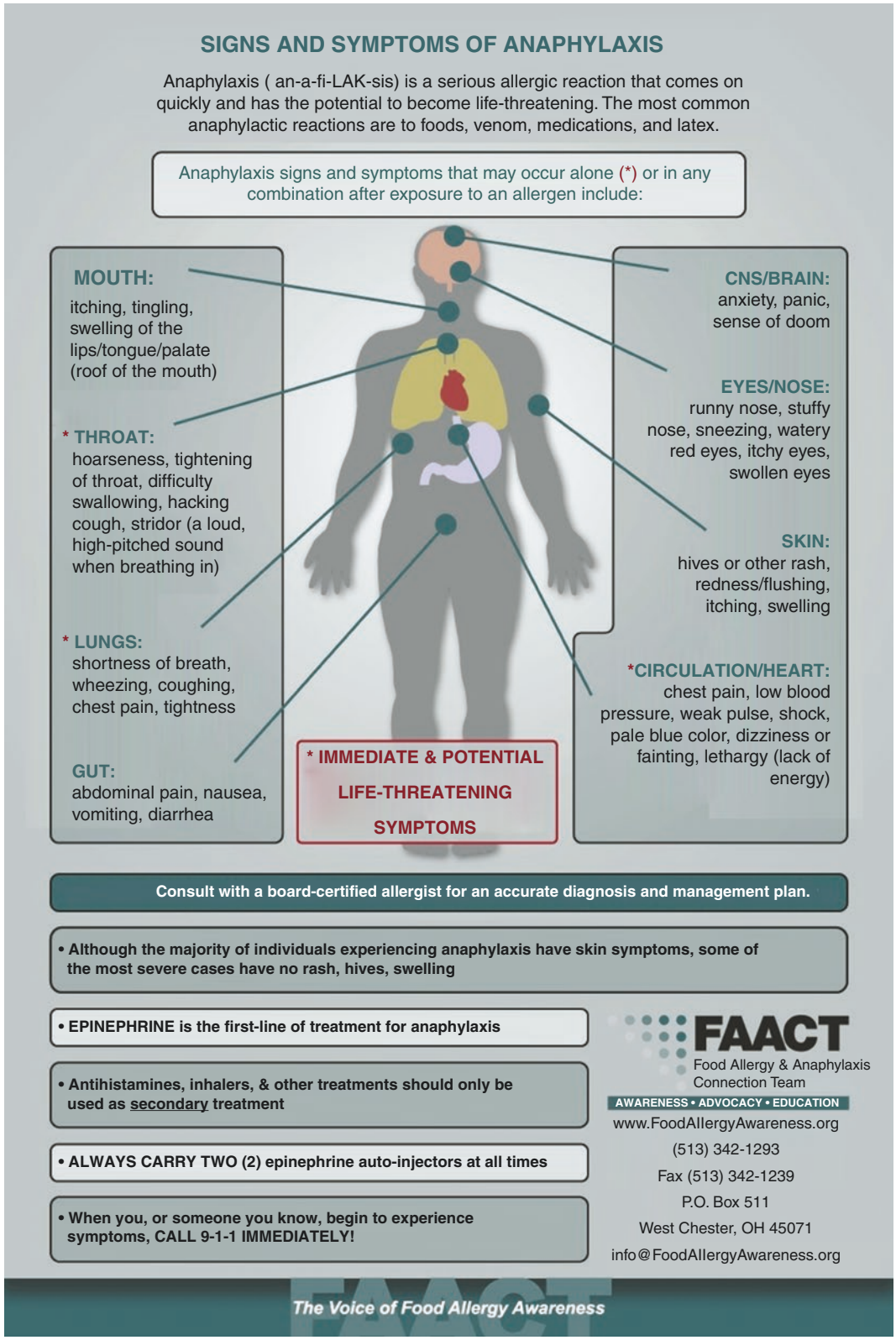

Fig. 16.1 Graphic provided and permission granted for use by FAACT. (Food Allergy Anaphylaxis Connection Team)

including in your diet all along to make restorative choices.

We also need to look at the places that are directly affected by the malfunction. Our stomach houses more neurons than our entire spine. Neurons are the messengers that ensure our body gets all of the correct signs of what to do and how to act. The connection between the brain, digestive system, and disease has been considered for many centuries [9]. Undiagnosed food intolerances are an easy highway to setting off the wrong signals. If your gut is not optimal, neither is the rest of you. It is very possible that the foods that you are eating are the root cause of many of your ailments. This may not be the case for everyone, but it is a theory that can be tested without the use of medications or the possibility of side effects from medications given as a symptom Band-Aid. I am in no way suggesting that medications should not be used—simply that there are alternatives that can be tried for those who are hypersensitive to medications and, therefore, may have adverse reactions, adding further symptoms into the mix.

One general term that has been used to explain insufficient gut health and how it affects the rest of our overall physical wellbeing is the leaky gut syndrome. To explain simply, when we do not eat properly or eat foods that are offending, the digestive system becomes damaged. The necessary substances that we would normally distribute throughout our bloodstream are malabsorbed and our usual functioning becomes disrupted. When our body does not filter what is necessary to maintain a healthy balance, we begin to see symptoms of the poor constitution. Although they may begin on a small scale including symptoms such as fatigue, indigestion, muscle weakness, and hard to diagnose ailments, over time, the deterioration can increase to overall body system damage that could potentially be irreversible. Poor gut health may also worsen diseases that a patient already exhibits such as allergies, asthma, celiac disease, arthritis, and more. If malabsorption can begin to break down the most basic of bodily functions, then there is proof that it can also attack a person's immunity in the process. "But a simple switch in foods means *the cause of the inflammation is removed* – and the body begins healing *naturally*" [10].

These symptoms can turn into a multitude of aches and pains that may seem like minuscule complaints to a doctor while causing unusual and possibly debilitating situations to those that are being affected by them. That which begins as a bit of something that seems to be off-balance can escalate to anything from increased allergies, weight gain, hypo/hyperglycemia and thyroid, acid reflux, muscles and joint pain, fogginess, and eventually the breakdown of physical and mental health. With your gut health being riddled and your adrenals being depleted, your body begins to run on empty. No good comes from allowing your body to constantly be depleted, exhausted, stressed, and hypersensitive. "Food intolerance has been associated with asthma, chronic fatigue syndrome and irritable bowel syndrome (IBS)" [11]. Those that have been undiagnosed for long periods of time may often find that they are in a state of adrenal fatigue. Adrenal fatigue is a general term used to describe many challenging symptoms that do not seem to fit one particular diagnosis. Chances are that if you have adrenal fatigue, your immune system is compromised even more which elevates the need to avoid any foods that will cause inflammation.

What Is Immunity

"The immune system is an organization of cells and molecules with specialized roles in defending against infection" [12]. A study published in September 2017 exhibits proof that two of our main body systems work together in ways that we may not have been associated together in the past. Medical professionals and scientists understand how the body sends messages out to try to heal itself, but factors discovered in this study point to a possible new method of treatment for those with inflammatory conditions and diseases. The information obtained from the study shows signs of a working relationship between cells within the gut itself. Not only do the neurons lining the gut send out messages of imbalance and inflammation but there are also innate lymphoid cells that seem to play a role. The lymphoid cells contain a receptor for a protein called neuromedin (NmU). While testing, scientists found that

this protein was released, causing the neurons and innate lymphoid cells to team up and try to heal the body faster. The study A Cellular Tango: Immune and Nerve Cells Work Together To Fight Gut Infections shares an interesting finding "Where we are most excited in thinking about multiple chronic inflammatory diseases that might be related to this neuronal-immune axis and where we might be able to intervene," Dr. Artis said. The findings may have important implications for scientists studying inflammatory diseases, including asthma, food allergies, and inflammatory bowel disease (IBD). Dr. Artis said it was too soon to say whether NMU itself or its receptors could be treatment targets, but he said studying these pathways might lead to potential new therapies for these diseases [13]. Indeed, if we can understand the who, what, where, and why of inflammation and how to control it, then our bodies could potentially be the superstar of modern health, playing the role of the interpreter who solves the mystery.

We have long heard the term *you are what you eat*. This terminology can be deciphered in multiple ways, depending on what your theory is on the food connection. "This may sound implausible to you, but the notion that good bacteria not only influence what your gut digests and absorbs but that they also affect the degree of inflammation throughout your body, as well as your mood and energy level, is gaining traction among researchers" [14]. One such hurdle is the possibility that we are thinking backward. Both patients and physicians can become limited in their thinking after long battles with symptoms that do not seem to be alleviated by any particular treatment in a short amount of time. Our society is very much caught up in the quick fix epidemic which can lead to ongoing, improperly diagnosed symptoms that could not be treated with a simple pill. Understandably, nobody wants to remain unwell for longer than they need to be, and practitioners are skilled in matching up treatments and prescriptions with a typical list of signs and symptoms. Medications can cause side effects which may also cause more symptoms, masking the original symptoms. In the myriad of visits and follow-up, both physician and patient may feel overtaxed by the burden of information overload with little to no results. When this happens, there may be a few outcomes:

1. The doctor may feel that the patient is overthinking their health maladies and, in turn, possibly causing additional complications (i.e. Hypochondria).
2. The patient may feel frustrated by the lack of sincere treatment options due to speculation that the maladies are occurring from what they think may be happening versus what is actually happening.
3. Either or both doctor and patient may decide not to continue their doctor/patient relationship because one or both of them feel adequate care, treatment, or information is not being given or is seemingly unavailable at that time.

It is possible that some symptoms manifest due to the stress of unknowing. However, the Hippocratic Oath is *first, do no harm*. This includes mental as well as physical harm; this oath applies to help those who insist they know their body better than the physician. Indications of illness are not always what the brain thinks they are. As written in the article The Pit in Your Stomach Is Actually Your Second Brain, the author stated, "Up to 90 percent of the cells involved in these responses carry information to the brain rather than receiving messages from it, making your gut as influential to your mood as your head is" [15]. Future thinking of diagnosis must be reversed—what is our innate health trying to tell us when we don't feel well? The affliction path is not from your brain telling your body parts what aches but your gut sending out the distress signals as it moves throughout the body from the messages.

Food Elimination Diet

Various testing can be done to try and pinpoint which foods are the most offending. Testing for food intolerances has been a bit of debate within the medical community as some are skeptical of the accuracy of the testing as well as people who claim that food intolerances are a real condition for them. "While the test seems to be growing in

popularity, no mainstream medical bodies have endorsed its use for diagnostic purposes, and scant reliable scientific evidence exists to support its utility in pinpointing food intolerances" [16]. However, "Could it be that, far from being in the vanguard of scientific discovery and development, the medical fraternity is, yet again, trailing behind while the patient and the food industry lead the way?" [17]. IgE testing is for a food allergy and is very different from an IgG test panel which indicates food intolerances. Typically, IgE testing has been performed to display the top eight food allergens that will cause an immediate reaction upon ingestion or contact. An IgG food panel can check for multiple offending foods and will give the patient a general knowledge of how much the exacerbating foods may contribute to their reactions. Tests typically include a value system of a class of numbers and/or categories for low, medium, and high for sensitivities. Keep in mind that IgG testing does not provide a definite map of which symptoms are caused by which foods. There is some legwork that has to be done to match the symptom to the food.

Testing

Usually by blood but sometimes through saliva. Ranges also vary upon age [18].

Result Interpretation

Delivered through a physician or consultant who can accurately interpret the results for the patient. Simply testing and not explaining the results will result in less accuracy in understanding the test and which foods to eliminate or minimize.

Guidelines

Providing guidelines to the patient as to how to not only eliminate the foods are causing irritation but also how to replace them to stay nourished and maintain satiety. "There is some suggestion that different protein sources differentially affect satiety" [19].

Follow Up

Provide follow-up and support to ensure the patient is comfortable with seeking replacement foods and answer questions. "Unfortunately, most physicians lack adequate nutrition training and resources, and they face many other challenges in delivering such information.

Barriers that challenge physicians in counseling their patients about nutritional change include lack of time, financial disincentives, competing agendas, a perception that nutritional counseling lacks effectiveness, lack of knowledge about nutrition, lack of training and expertise in lifestyle modification techniques, and uncertainty about changing guidelines. The lay public also is confused about which dietary recommendations should be followed" [20].

Retest

Retesting is recommended, but I advise not to do so before 6 months, with the consideration of waiting even longer until the patient has eliminated all of the offending foods. This avoids possible inaccurate/duplicated test results on foods that have not yet been eliminated and reduces the out of pocket cost for the patient.

Goal

The goal of eliminating and replacing offending foods is for the patient to treat the cause of their symptoms and heal their gut. An additional factor to include in the testing and elimination as mentioned by The Mayo Clinic "One of the tricky aspects of diagnosing food intolerance is that some people are sensitive not to the food itself but to a substance or ingredient used in the preparation of the food" [21]. As discussed in the previous paragraphs, a healthy, happy gut will then send out healthy, happy messages to the brain and should decrease the irritating symptoms. Very specific nutritional help should be offered, and I strongly recommend utilizing a person that specializes in foods beyond just the Nutrition or Dietician aspect. Patients working with a

Nutritionist or Dietician may receive some helpful food information, but it will be loosely based on fat, calorie, etc. content rather than foods that are or are not allowed for the person that they are counseling. They may give generic meal plans and they may or may not offer support to a certain extent, but having a wide range of food allergy and intolerance knowledge is the key.

Avoidance

Why should someone avoid foods for a specific amount of time? To improve immunity, you must allow time for your antibodies to heal in order to avoid the continuation of reactions. It can take your body anywhere between 21 and 23 days to rejuvenate. If the patient continues to consume some of the highlighted foods, this will continue to deplete the body of necessary healthy antibodies. In addition, it will continue to strip away the stomach's primary functions (absorbing necessary fats, proteins, and nutrients) which will filter through the body on a less than optimal level. Continued levels of malabsorption will lead to other system breakdowns. The longer your stomach neurons are damaged, the longer it will take to repair.

Imagine the neurons in your stomach were fed through a straw. As long as there are no kinks or blockages, all of the benefits that you draw from your food travels from point A to point B. Next, poke a few holes in the straw. Your body is still getting some or most of what it needs but it's still lacking the full capacity of what keeps your systems functioning well. It may not be completely damaged but it's also not completely and accurately doing what it should be doing. The patient may notice some symptoms but assume they are associated with outside factors (such as viruses, environmental changes, stress, sleep pattern changes, poor food choices, or hormonal changes). Once malabsorption begins, our overall immunity and nutrition follow in a downward spiral. A body that lacks nutrients cannot ward off disease as quickly or as fiercely. The larger the amount of aggravating foods, the larger the amount of less or nonfunctioning cells within our immune system.

Within the years that I have been referred client consultations, I have seen how foods can improve various health issues. Is food the answer to everything? No, but it's the place that is commonly overlooked and yet the easiest place to begin.

Healing

Simply put "When the cause of malabsorption is treatable, the primary goal of treatment is to treat the cause" [22]. Avoidance of the foods is just one of the steps to getting your gut health back on track which will allow better immunity as well. For some, full avoidance is unquestionably necessary and it will decrease the amount of time that it will take for healing to happen. For others, it includes a rotation diet of some of their foods. A rotation diet is when a person makes an effort to space out their foods rather than eating the same foods every day (ex: someone who has a banana with breakfast every morning would rotate it and have one banana on a Monday, then one banana on a Thursday). Rotating foods gives our system a period to calm down. I do caution that a rotation diet is not what is best for everyone. Rotation diets are best considered with foods that are highlighted on the lowest end of the food intolerance test and even then, it may depend on the food(s) due to how other foods can continue to feed the imbalance inside of your stomach. Typically, a rotation diet may be a less upsetting introduction to a patient who feels that complete elimination is just not within their realm of power. Should you discuss a rotation diet, it is imperative that you also include the factors that the patient may not consider; it will take a longer time to heal, it will be a bit more complicated to understand which foods are causing which ailment and it may not give them the immediate health boost that they picture happening as a quick fix. There is always the possibility that specific foods may need to be avoided going forward to decrease or eliminate symptoms that continue when they are reintroduced.

Food Diary

Keeping a food diary is cumbersome but can be motivating, especially with those who need to

have some type of proof of what is signaling their body to be out of sorts. It is very difficult to keep track of what we eat every day and what we felt after we ate those foods. By keeping a notebook of everything that we put into our bodies, any and all symptoms (even if it is seemingly unrelated) and the days that the patient felt them, one could easily trace symptoms back to foods. "A diary or journal is a way to map your symptoms and see if there is a relationship to the foods you have eaten. It should always be done with the help of your doctor. Diaries can be an excellent way to start exploring puzzling symptoms and often help to show that foods are not the cause" [23]. This may seem rudimentary, but it is effective as long as the patient is consistent with the note-taking. It is, as I repeatedly mention, a method that causes no harm or side effects to the patient and costs virtually nothing.

Remind your patient to be patient! Everybody's body heals differently and nothing will be healed overnight. Your patients look to you with their lives in their hands—treating them as anything less means that you are not fulfilling your oath. Explain how their immune system works in terms that they can easily understand. Not everyone knows that their antibodies need a vacation to get back to work or that doing too much at once may have also caused a Herzheimer reaction, making them feel as if what they are doing to get better actually made them feel worse temporarily.

Case Studies

A woman in her 30s came to me for counsel. She very clearly displayed signs of anxiety and stress, was slightly overweight, and complained of chronic fatigue and horrific brain fog. She stated her ability to perform simple, everyday activities had diminished and felt it was difficult to function even with the simple responsibilities of the day-to-day. She also made it a point to tell me that she did not cook nor did she eat fruits or vegetables. Her food intolerance test panel displayed elevated results for dairy and gluten, among others. Her yeast was also high, and this presented not only a food restriction for a food group but also an additional barrier in which foods she should eat and avoid as well as which foods to specifically not eat as they would increase yeast growth in her gut. The biggest challenge for her was how to get the proper foods, prepare them and still accomplish what needed to be done in the limited hours of her family's schedule. It is imperative that everyone understands that taking care of oneself is always a necessity. It is crucial to know that keeping your own health a priority is not a selfish act and it's not something that should be written off by anyone, not even yourself.

Some months later, that person came to visit me at a local event. She was bright-eyed, smiling, and had bountiful energy. Her eyes looked clear and hopeful. She had lost weight and had regained her energy levels. She was even eating foods that she had not eaten in the past. She admittedly said the beginning was difficult for her, but the better she ate, the better she felt. She shared that her anxiety had almost diminished. Some physicians may call this a placebo effect; however, a placebo is known to "fix" the side effects, but they are not able to heal or cure the problem. Therefore, if a patient is healing—actually healing and thriving from making specific food choices—is that not a treatment versus a placebo?

A male food allergy consultation agreed to share his story as well to help others understand how simple food changes can, indeed, bring a difference to your health and how you feel overall. His symptoms began with seasonal affective disorder. After consulting with an alternative physician, it was suggested that he try removing gluten, dairy, and sugar. Upon removing the foods, he felt a vast difference. Symptoms would last 7 days on average when trigger foods were ingested. He describes it as coming on like a fog, gradually. It would take a couple of days to understand what was happening. Thoughts from previous medical professionals about his symptoms included that he was told "it was in all in my head. I had one gastrointestinal tell me I just needed to start eating again. They all wanted to prescribe medications for symptoms. But they had no idea about food intolerances and how much the stomach was involved with the functions of the whole body." – Steve B. To date, he reported that the symptoms have gradually lessened over time upon removal of the foods that

spurred symptoms. It also seems that because he has remained free of gluten and dairy as much within his ability, the symptoms upon accidental ingestion do not last as long. Specifically, with gluten, symptoms usually last 1–2 days compared to 7 days or more.

The key to begin to heal yourself is to know that your body takes as long to heal as it did to gain the damage sustained. There is nothing that will magically transition you to the healthier you. Not everyone has the same food intolerances; therefore, everyone should always be looked at as a unique case. Especially with food intolerances, trying to treat someone with the wrong foods can cause equally as much damage as not treating them at all. Food intolerances are trial and error, but the end results will amaze and astound anyone.

Food Intolerance Check Up

Rather than treating one symptom, treat the patient with a whole health plan. In lieu of medications or prescriptions that may add additional symptoms, first exhaust all other methods. This in itself also enables each patient to feel like a person treated as a person and not just a number fit into a time slot.

Beyond Blood Work

The worst thing to tell a patient who is unwell is that all of their blood work is "within normal range" when, in fact, it's only based upon the range of blood work that you are willing to check. Check everything, even if you don't normally agree with that type of testing (this includes checking antibodies, etc.).

Test for Food Allergies and Sensitivities

Without having every piece of the health history, you are only working on a small piece of your patient's symptom and not the cause. All of these can be treated together and on an individual's specific level versus limiting it to prescribed generic written prescriptions (i.e. food pump inhibitors).

Understand Your Limits

If you are part of the group of physicians that don't necessarily agree with out-of-the-box thinking, then, at least, be willing to begin the journey with your patient, but also be professional enough to let them know you may not be the best resource for them to finish with. Not every doctor specializes in every health aspect; be a better person to yourself and your patients and refer them to those who can help them.

Do Research

No eye-rolling, just roll up your sleeves and do it. You may not agree, understand, or accept it even after you have researched these newer methods, but finding the causes of your patient's concerns and health issues are never about you—it is always about the patient.

Conclusions

Over the years, I have counseled people who went through years of not knowing the cause of their illnesses and symptoms. I am a firm believer that our bodies tell us far more than what doctors can pinpoint through traditional scientific study, treatment, and procedure. I believe that the way that our foods have been modified over the years, and how/what we eat directly affects the way that we feel. Client after client, I have seen a person's life change simply by eliminating or minimizing offending foods. Symptoms have ranged and/or included but are not limited to severe fatigue, muscle aches, stomach issues, brain fog, hair loss, mood and behavioral changes, low sex drive, and more. I have been updated as a person's life has

improved and symptoms that had long been assumed something that they created inside of their head were in fact linked to food(s).

It is my opinion that food intolerances are a real cause for some, not all. I also feel that the puzzle of food intolerances and how they directly affect our nutrition and immunity are undeniably intertwined. It is not necessarily which is the initial cause but which area we look at to support all of them as they all support how a person functions properly. I feel that the mystery of how people's health began to increasingly struggle and/or fail is due to multiple factors but especially those dating back to the introduction of genetically modified foods and/or chemically-stabilized foods being the norm versus incorporating whole foods is a direct component to all of our lowered immunity. In my opinion, ongoing intolerances may also be stemming from the changes in food guidelines which incorporate more carbohydrates and grains versus ancient grains. An additional subject that needs to be continued to be studied is the debate which challenges this guideline, stating the human stomach does not digest specific foods, thus leading to continued damage of the systems connected. Those who are in a capacity to help others must consider utilizing all thoughts of medicine and healing, not simply conventional medicine.

Most importantly, it is admirable to admit that you do not have all of the answers. It is even more admirable to let others know that *not* knowing doesn't mean you will not seek to help them. Support all theories until you have proof that they are false or unusable for each person that they may present with. Reinforce what you have been taught, but continue to be open-minded so that you may bring your own knowledge as a physician to a level that was not taught to you. Most patients will not remember the doctor that did not help them, but they will remember the doctor that stuck with them and did help them. When it comes to who you treat, any person that puts their faith into your hands chooses to be the one that restores them to their wholeness. "Never worry about numbers. Help one person at a time and always start with the person nearest you." Mother Teresa.

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17 Potency of T-Cell Epitope-Based Peptide Vaccines in Food Allergy Treatment

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Key Points

- Allergen avoidance or drugs that aid symptom relief are currently the only options available for food allergy treatment.

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- Allergen immunotherapy (AIT) can be the treatment that really tackles the cause of allergy.
- The potential of AIT with whole allergen extracts for food allergy is often questioned due to safety, efficacy, and compliance issues.
- Food allergies result from an imbalance between different T cell subsets.
- AIT with T cell epitope-based peptide vaccines may provide a better alternative in treating peanut allergy than conventional AIT with whole allergen extracts.

Introduction

Continuous feeding of a protein was shown to prevent the induction of an immune response to the same protein [1]. In addition, prior feeding of the antigen inhibits the immune response to a systemic antigen challenge [2]. This phenomenon is called oral tolerance and is the result of an active regulatory response by the immune system [3, 4]. However, in food-allergic patients, the induction of oral tolerance to food proteins is disturbed. A combination of multiple factors, such as genetic predisposition, route of exposure, allergen dose, and structural characteristics of the allergen, may alone or in combination be responsible for allergy development [5]. Typical food allergies are IgE-mediated, and symptoms develop within minutes to 1–2 hours after food ingestion. These manifestations derive from a failure to develop or a breakdown of food tolerance, resulting in excessive production of food-specific IgE antibodies and in altered cellular T-cell events, leading to allergic reactions.

T Cell and Food Allergy

T-Cell Activation and Differentiation

T cells are a subtype of white blood cells and include $CD4^+$ helper T (Th) cells [6]. Activation of naïve T helper (Th0) cells occurs by antigen-presenting cells (APCs). Although there are several subsets of APCs (including dendritic cells (DCs), macrophages, and B cells), DCs are the only APCs that can induce T-cell proliferation of naïve T cells [7–9]. DCs are located at the site of antigen entry. After DC-dependent phagocytosis of the antigen, the antigen will be digested into smaller peptides. These peptides are presented on the cell surface of DCs by major histocompatibility complex (MHC)-II molecules [8, 10]. The first signal for T-cell activation results from the interaction between the T-cell receptor (TCR) on the T cell and its specific peptide in complex with an MHC-II molecule on the DC. Besides antigen recognition, the presence of specific costimulatory receptors and the presence of cytokines during early immune responses are crucial for proper T-cell activation [11]. The formation of a TCR-peptide-MHC-II complex is the first requirement for Th0 cell activation. Since CD4 coreceptors on Th0 cells are important in facilitating the TCR-peptide-MHC-II complex interaction by binding to MHC-II molecules, the presence of the CD4 coreceptor is also crucial for proper Th0 cell activation [6]. CD28 is considered the most important costimulatory receptor for enhancing TCR signaling and inducing T-cell activation [12, 13]. The costimulatory receptor CD28, which is constitutively expressed on Th cells, interacts with CD80 and/or CD86 on DCs upon activation by the antigens they encounter. Several other interactions between receptors on DCs and receptors on T cells are found to be involved in T-cell activation [6, 14]. Food allergies are caused by an imbalance of specific T-cell subsets.

The combination of recognition of peptide-MHC-II complexes by the TCR and interaction between coreceptors on T cells and DCs induces the secretion of cytokines by (primarily) DCs. The secretion of these cytokines provides the third requirement for proper T-cell activation. Cytokines are soluble proteins that act as chemical messengers in the communication between immune cells, thereby causing immune responses. Various types of cytokines exist, including several interleukins (IL), interferons (IFN), and tumor growth factors (TGF) [6]. IL-2 is an example of a cytokine that illustrates the significance of cytokine secretion in proper T-cell activation. Early after antigen recognition and costimulatory receptors interactions, the secretion of IL-2 (by

Th cells in particular) is initiated. This cytokine, known as the central T-cell growth factor, is very important for the growth, survival, and differentiation of Th cells. By secretion of this cytokine during early phases of an immune response, Th cells promote their own differentiation and differentiation of Th cells in their close environment [12, 15]. The production of cytokines by DCs and some other leukocytes during the early immune response provides the third key signal needed to activate naïve T cells [16].

Different subsets of Th cells exist, including T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 (Th17), and regulatory T (Treg) cells [17]. A Th0 cell can differentiate into a Th1 cell by secretion of IL-12 by DCs and macrophages. This results in polarization to Th1 cells which secrete mainly IFN-γ. Alternatively, a Th0 cell can differentiate into a Th2 cell by the secretion of IL-4 by DCs, basophils, or mast cells. Th2 cells predominantly secrete IL-4, IL-5, and IL-13 upon activation. The secretion of TGF-β and IL-10 by DCs induces a Th0 cell to become a T_{reg} cell that predominantly secretes cytokines IL-10 and TGF-β [6, 18]. In contrast to Th1 and Th2 cells, T_{reg} cells have an immunosuppressive mode of action [18]. In a healthy situation, there is a certain balance between these different T-cell subsets [19]. However, in the case of (food-) allergic diseases, the imbalance between these different T-cell subsets appears as a result of reduced Treg cell compartment and expanded Th2 cell compartment [18, 20]. This imbalance results in a failure to establish immunological and clinical tolerance to the (food) allergen of interest.

Oral Tolerance

Several mechanisms have been described for oral tolerance, including anergy or deletion of antigen-specific T cells and the development of Tregs [21, 22]. It is known that antigen in high doses induces T-cell anergy/deletion, whereas in low doses it induces Tregs. However, it has been suggested that both mechanisms might occur simultaneously. T-cell anergy/deletion is induced when T cells are activated without co-stimulation [21, 23]. In addition, a role for Fas-mediated apoptosis has been described in T-cell depletion [23]. On the other hand, Tregs are induced after antigen presentation by immature APC, repeated exposure to antigen, or exposure to IL-27, IL-10, retinoic acid, and/or TGF-β [24–26]. There are several inducible Tregs described, namely $Foxp3^+$ Tregs, IL-10-producing regulatory type 1 (Tr1) T cells, TGF-β-producing Th3 cells, and $CD8^+$ Tregs [21, 22]. It has been shown that the induction of $Foxp3^+$ Tregs is favored by certain subsets of dendritic cells (DCs), such as $CD103^+$ DCs and $CD8^+$ plasmacytoid DCs, which produce TGF-β and retinoic acid [27–29]. Inducible Tregs may inhibit immune responses via several mechanisms [25, 30]. They may directly kill target cells via granzymes or may disrupt the metabolism of effector T cells by generation of adenosine or by depriving the cells of growth factors [25, 30]. Moreover, Tregs may secrete IL-10 and TGF-β that affect multiple immune cells and decrease cytokine production, mediator release, IgE production, and antigen presentation [25, 30, 15]. In addition, Tregs may inhibit effector and/or APC via cell-cell contact. The mesenteric lymph nodes (MLN) are an important site for tolerance induction. Worbs et al. have shown that removal of MLN almost completely prevented oral tolerance [31]. Moreover, they showed that tracking of DCs from the lamina propria to the MLN is important for tolerance induction.

Food Allergy

If oral tolerance to food proteins is not established or existing tolerance is broken down, this may result in a food allergy [21]. Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly upon exposure to a given food [32, 33]. In IgE-mediated food allergy, this immune response consists of two phases; the sensitization phase and the effector phase [34]. During the sensitization phase, APCs take up the allergen, process it, and present the peptides on MHC class II molecules to naïve T cells. These naïve T cells differentiate into Th2 cells, which produce pro-inflammatory cytokines, such as IL-4, IL-5, and IL-13. The cytokines in combination with the food allergen induce a class-switch in allergen-specific B cells. As a result, the B cells produce allergen-specific

IgE antibodies that bind to the high-affinity IgE receptor on mast cells and basophils. During the effector phase, the allergen binds to the allergen-specific IgE antibodies on the effector cell and cross-links them, thereby activating these cells. Several mediators, such as histamine, leukotrienes, prostaglandins, and cytokines, are released. These mediators act on epithelial, endothelial, and smooth muscle cells and thus induce acute allergic symptoms [10]. In addition, the mediators attract and activate other immune cells, such as eosinophils, which may induce late allergic symptoms. Although the majority of food allergies and/or food allergy-related adverse reactions are due to allergen-specific IgE antibodies and Th2-mediated immune responses, in some cases food-allergic reactions occur without clear Th2 and IgE involvement [7, 35].

Cow's Milk Allergy

Cow's milk allergy (CMA) is the most prevalent food allergy affecting 0.3–3.5% of the young children. However, 60–75% of the children with IgE-mediated CMA spontaneously develop tolerance before the age of 5 [36–38]. Still, these children have an increased risk of developing other atopic disorders later in life, being mainly asthma and rhinoconjunctivitis [36, 38]. In adults, the prevalence of CMA is much lower (0.1–0.3%), and they develop the allergy at adult age [38–40]. Allergic manifestations in CMA patients involve the skin, the gastrointestinal tract, the respiratory system, and the cardiovascular system with anaphylaxis in some patients [41–43]. In children in the UK, 10% of the fatal/near-fatal anaphylactic episodes were due to CMA [42]. CMA patients may be sensitized to all milk proteins, though αS1-casein and β-casein from the casein fraction and α-lactalbumin and β-lactoglobulin from the whey fraction seem to be most allergenic [44–46]. The frequency of sensitization for the different proteins varies between different studies and depends on the patient population and the methods used to determine IgE levels. Principally, there is no curative treatment for CMA, and avoiding exposure to cow's milk appears the only sensible option [42, 43]. However, as cow's milk is currently found in many foods, and with accidental exposure to cow's milk occurring frequently, this restriction has a great impact on the diet and quality of life of CMA patients and their relatives [47–50].

When breastfeeding is not possible, cow's milk is an important source of nutrition in young children (<2 years old), and therefore, substitute hypoallergenic formulae based on hydrolyzed milk proteins have been developed [36, 41, 44]. Hydrolysis is generally based on enzymatic degradation of casein and/or whey proteins and based on the degree of hydrolysis and the length of the remaining peptides; these hydrolysates are (arbitrarily) categorized as partial or extensive [51–53]. As partial hydrolysates still contain larger fragments and thus can induce allergic symptoms, they are not suitable for all treatment purposes [52, 53]. Extensive hydrolysates only contain small peptides and are in general well tolerated in CMA patients, except for the very severe ones [43, 53, 54]. The World Allergy Organization recommends extensive hydrolysates for the treatment of CMA children with a low risk of anaphylactic reactions, whereas for the high-risk ones, formulae containing free amino acids are recommended [55].

Currently, it is debated whether exposure to allergens is a prerequisite for allergy development and it is still wide practice to recommend allergen avoidance for prevention of allergy [56–60]. Also in children at risk to develop CMA, substitute formulae have been used. As partial hydrolysates generally contain larger fragments, being more immunogenic, it was tested whether this partial hydrolysate may actually prevent CMA by inducing tolerance to cow's milk [41, 51, 61–63]. Indeed, clinical studies have indicated that both partial, and extensive hydrolysates, may prevent CMA and atopic dermatitis in high-risk children [53, 54, 62, 64–68].

Peanut Allergy

The prevalence of peanut allergy has doubled over the past two decades, nowadays affecting approximately 1–2% of the population [21, 22]. In contrast to most food allergies, peanut allergy

is highly persistent and only about 20% of peanut-allergic children are expected to outgrow their peanut allergy [22, 69]. Besides the fact that peanut allergy is often a lifelong condition, it is also the most common cause of fatal anaphylaxis [21, 22]. Since a cure is not yet available, standard care is strict peanut avoidance, together with direct access to drugs that aid symptom relief. The observed increase in peanut allergy among children (from 0.6% in 1997 to 2.1% in 2008) is poorly understood. In both Europe and the US, virtually all children are exposed to peanuts by the age of two [70, 71]. Due to the relative immaturity of the infant immune system, immunoreactivity to food is more likely to develop during the first few years of life. Importantly, increased exposure throughout childhood also induces increased peanut sensitization in genetically predisposed children.

Despite the current standard approach of peanut allergy management, still, 50% of peanut-allergic patients report accidental exposure, illustrating that avoidance is not a long-term solution [24]. Moreover, depending on the severity of symptoms, the quality of life of peanut-allergic patients may be severely affected due to high levels of anxiety and the continuous dietary and social restrictions these patients are subjected to [25]. Because of the reported constraints, there is an urgent need for new types of peanut allergy treatment besides antihistamines, bronchodilators, and adrenaline injection for emergency treatment of anaphylaxis [23].

Immunotherapy

Conventional Allergen Immunotherapy

Since food allergy is considered to result from a failure to establish immunological and clinical tolerance to specific food proteins, expectations are that obtaining that tolerance leads to resolution of food allergy [72]. To date, allergen immunotherapy (AIT) is the only type of treatment that is really aiming at altering the allergen-specific immune response in allergic individuals towards a state of tolerance [6, 23, 73]. The goal of AIT is increasing the threshold of allergen needed to induce an allergic response. This goal is established by repeated exposure to progressively increasing allergen doses, followed by stable maintenance doses of the allergen, over a period of weeks to years [23, 21, 27].

Both desensitization and sustained unresponsiveness are important concepts in AIT. Desensitization is defined as the capacity to tolerate a higher threshold amount of allergen without allergic response in allergic individuals receiving AIT [74, 75]. Sustained unresponsiveness (permanent tolerance) is referring to the capacity to retain this tolerance to a higher threshold of allergen after AIT has been canceled for weeks up to years [74, 75]. Desensitization to an allergen following AIT does not necessarily result in permanent tolerance eventually. In contrast to permanent tolerance, desensitization may be transient. This indicates that the higher threshold amount of reactivity is only maintained upon regular exposure to the allergen by AIT [22, 76]. The ability of peptide vaccines to induce desensitization or sustained unresponsiveness needs to be assessed by determining the doses an allergic patient successfully tolerates in a double-blind placebo-controlled food challenge (DBPCFC) [77].

AIT is proven to be effective for allergies to many aeroallergens and insect venom and is, therefore, an accepted type of treatment for these allergies nowadays [23]. Although both different delivery routes and different forms of allergens have been tested in AIT trials, only whole allergen extracts are used in clinical practice, with subcutaneous immunotherapy (SCIT) as the most effective route [78, 74]. Despite the efficacy of conventional AIT seen for treatment of inhalant and insect venom allergies, it is currently not used in clinical practice for the treatment of peanut allergy or other food allergies [27]. This is a result of ongoing concerns regarding safety and efficacy of SCIT, and low compliance to SCIT and other types of conventional AIT in food allergy treatment.

To date, AIT is the only type of causative treatment that is aiming at altering the allergen-specific immune response in allergic individuals towards a state of tolerance [6, 73, 79], which is intended to establish sustained immunological

and clinical tolerance to these allergens after the therapy ends [26].

Whole allergen extracts are still used in conventional AIT in food allergy treatment and contain intact immunoglobulin (Ig)E-binding epitopes that can cross-link effector cell-bound IgE molecules [80]. The presence of these intact IgE-binding epitopes increases the risk of severe systemic adverse responses during therapy. Therefore, the safety of conventional AIT in food allergies (such as peanut allergy) is often questioned. In addition, the use of whole allergen extracts is associated with certain safety problems. Especially from natural allergen extracts, it is questioned whether unidentified epitopes are still present in the extract. These may be epitopes to which the patient is not sensitized yet, but may become sensitized to during treatment [75]. In the case of peanut allergy, treatment with vaccines containing natural allergen extracts may lead to an increased risk of severe adverse responses in the future. This increased risk is the result of the introduction of these novel specific IgE subtypes to a peanut-allergic individual's IgE repertoire [21, 81]. Additionally, doubts are present about the capacity of conventional AIT to induce desensitization and permanent oral tolerance to peanut allergens thereby limiting vaccine efficacy [27, 79, 82]. The adherence of peanut-allergic individuals to conventional AIT is expected to be low due to the development (or the risk of) adverse responses during treatment, trouble with adhering to the described doses, and/or of the need for a prolonged treatment period for achieving successful desensitization [27, 77, 79, 83]. Since the possible effects of discontinuation of conventional AIT have not been completely addressed yet, precautions should be taken when considering the practical applicability of conventional AIT in peanut allergy treatment [76]. Therefore, further research is needed to evaluate to what extent conventional AIT may be useful for peanut allergy treatment and how its safety, efficacy, and adherence can be improved [27].

In the quest for a potential future cure for peanut-allergic diseases, the focus should be on examining which treatment has a high immunogenicity (capacity to induce Treg/Th1 and IgG4 blocking antibodies), but a low allergenicity (IgE cross-linking capacity on basophils and mast cells) [18]. In other words, the treatment should have a retained ability to induce desensitization and permanent tolerance, but a reduced ability to induce allergic responses in allergic individuals [79, 84]. Attention should be particularly paid to the factors that drive allergic responses versus those that drive tolerance. As a Th2-skewed pattern of cytokine production is observed at sites of allergen exposure in food-allergic individuals, downregulation of these allergen-specific Th2 cells may relieve allergy. In addition, an increased expression of regulatory T (Treg) cells is required, since the induction of this immunosuppressive T-cell subset is considered to be essential for inducing immunological tolerance [18]. This indicates why a T-cell-targeted approach may be useful in food allergy treatment. T-cell epitope-based peptide vaccines' identification of suitable allergen preparations for specific immunotherapy requires detailing characterization of sites that interact with antibodies or B cells and T cells. T cells interact with linear peptides presented in the context of MHC molecules on APC surfaces. It has been suggested that children with milk allergy whose predominant IgE reactivity is against conformational epitopes are more likely to develop tolerance to milk than those who react against linear epitopes, but whether this relationship occurs for peanut allergens is not yet known [85].

The potential of peptide vaccines in allergy treatment depends on their composition of short synthetic allergen peptides that contain dominant T-cell epitopes [20, 23, 79]. These short synthetic allergen peptides consist of sequences that are too short to cross-link allergen-specific IgE on mast cells, basophils, dendritic cells (DCs), and B cells. Therefore, their potential to induce mast cell and basophil degranulation (thereby triggering allergic responses) is negligible, just like their ability to induce the production of inflammatory mediators by DCs and B cells. The vaccines are produced in a standardized way so that the composition of the vaccine can be accurately controlled and the allergenic potential of these peptide-based vaccines can be better controlled than that of natural allergen extracts [80]. The

relatively low allergenicity of these peptide vaccines, compared to conventional AIT, may lead to an improved safety profile for the treatment of severe food-allergic diseases (such as peanut allergy).

Although the allergenicity of peptide vaccine treatment is considered to be lower than that of conventional AIT, they still retain their immunoregulatory capacity and also their ability to make peanut allergen-specific T cells unresponsive to future allergen exposure (desensitization) [27, 84]. The capacity of peptide vaccines to induce sustained unresponsiveness was even found to be higher than that of conventional AIT [20]. This is most likely due to the fact that the lower allergenicity of peptide vaccines allows for high-dose allergen administration, which is required for obtaining a significant immunoprotective effect [86]. This possibility of high-dose allergen administration in peptide vaccine treatment may also significantly improve treatment adherence compared to conventional AIT. In contrast to peptide vaccine treatment, conventional AIT has to deal more often with dose limitations in order to prevent IgE-mediated allergic responses. These dose limitations indicate why a long treatment course is often required for establishing successful desensitization [87]. Results from studies testing peptide vaccines in allergic individuals indicate that only a short period of treatment is required to reach a state of tolerance and thereby peptide vaccines may provide a better alternative for treating food-allergic individuals [23, 79, 87].

Altered Peptide Ligands for Immunotherapy

T-cell targeted therapies have been developed aimed at downregulating the aberrant allergic response. Dominant T-cell epitopes containing short synthetic peptides have been developed that bind specific HLA class II alleles providing sustained immunological and clinical tolerance. The mechanisms underlying their applicability include induction of anergy and/or clonal deletion, and immune deviation and Treg induction. These mechanisms rely on the functional cytokine plasticity of Th cells to downregulate pathogenic and allergic effector T-cell responses. Repertoires of T-cell epitopes of allergens are conserved for at least 2 years in allergic individuals, and the clonal T cells involved have the ability to switch from dominant IL-4 production to dominant IL-10 or IFN-γ production during conventional AIT [88–90]. Analysis of the human T-cell repertoire reveals a bias in both the TCR-Vα and TCR-Vβ gene segment usage. When applied in experimental systems and clinical conditions associated with allergic rhinoconjunctivitis, this T-cell epitope peptide therapy appears to be safe and effective, with the potential for application in more severe allergy cases and in food allergy [91–94].

To identify all potential T-cell epitopes, allergen-specific T-cell lines and clones generated from a large patient cohort are screened for reactivity against overlapping synthetic peptides spanning the entire sequence of the allergen molecule, each usually 15–20 amino acids in length with overlaps ranging from five amino acids upwards. Following identification of T-cell reactive peptides, precise core epitope sequences are mapped utilizing peptide sets truncated from the N- and C-termini, for example, as demonstrated in early studies for a ryegrass pollen allergen Lol p 5 T-cell epitope [95, 96]. However, more recently systematic approaches to this are being utilized, including peptide microarray platforms and more readily available MHC class II tetramers, which may facilitate future epitope identification [97, 98].

Minimal core CD4+ T-cell epitopes are typically eight or nine residues long, but lengths for optimal T-cell stimulation may be longer and vary between subjects. This likely reflects varied requirements for flanking residues in stabilizing different HLA-peptide-TCR complexes and increasing the persistence of the peptide at the APC surface [99–101]. Peptides selected for immunotherapy tend to range from 12 to 20 residues, consistent with naturally processed peptides eluted from HLA class II molecules. T-cell epitopes are typically found throughout an allergen sequence, but responder frequency evalua-

tions from large subject cohorts assign dominance [102], underpinning design of T-cell-targeted peptide therapeutics. Dominant T-cell epitopes also typically have the strongest T-cell stimulatory capacity, an important consideration for immunotherapy following the established immunological dogma that the strongest immunogens are the strongest tolerogens [103].

Some peptides require modification to ensure solubility and stability for ease of manufacture and administration. This may include modification of terminal residues and substitution of cysteine residues with alanine or other nonreactive residues such as serine to avoid potential peptide aggregation [104]. In these cases, T-cell reactivity of the modified peptide must be reconfirmed. The name altered peptide ligand (APL) was coined to better describe antigen-derived peptides bearing single amino acid substitutions that stimulate some, but not all, T-cell functions. With APL they were referring to those analogs of the wild-type, immunodominant peptides, in which distinct TCR contact residues had been structurally modified by usually conservative single amino acid substitutions. An early study showed that ovalbumin peptide delivered intravenously was able to tolerize peptide-specific CD4 T cells, while the same peptide administered in complete Freund's adjuvant via a subcutaneous site generated peptide-specific memory T cells [92].

So, while the effector function, measured as cytotoxicity or cytokine release, does not generally diminish with increasing peptide concentration, the ability of the T-cell clone to divide and expand can be compromised. For some closely related allergens, for example, group 1 grass pollen allergens, cross-reactive T-cell epitopes have been identified which may be advantageous for obtaining broader-acting therapeutics with applicability in cross-reactive food allergies [105]. However, APLs encompass a collection of antagonists, partial agonist, as well as putative superagonists of the immunodominant wild-type peptide [92], while still others without any clearly identifiable activity were revealed. This makes applicability in food allergy treatment still subject of further research.

Peptide Immunotherapy

Peptide Therapy for Cow's Milk Allergy

Ideally, therapy for CMA should induce long-lasting tolerance without activating mast cells and basophils by inducing T-cell anergy or Tregs. However, this can only be achieved when T cells are activated via their T-cell receptor without co-stimulation or in the presence of immunosuppressive cytokines, such as IL-10 and TGF-β [3, 106, 107]. T-cell receptors recognize peptides of 9–12 amino acids long, which are much smaller than the peptides that are needed to cross-link IgE antibodies bound to receptors on basophils and mast cells (minimal 35 amino acids) [108]. Peptides that are too small to cross-link IgE but long enough to induce T-cell activation might thus be a safe alternative for conventional immunotherapy.

So far, the efficacy of T-cell peptide immunotherapy has mainly been shown for inhalation allergies in mouse models and with 10–17 amino acid long peptides in cat and bee venom-allergic individuals by administering a mixture of peptides (10–17 amino acids long) intradermally or subcutaneously [109, 110]. Four injections of a peptide mixture decreased allergic symptoms even 9 months after the therapy was stopped. Generally, the treatment reduced the allergic symptoms without acute side effects, although late phase allergic symptoms can occur that decrease during treatment [111, 112].

To date, a limited number of studies have investigated the potential of peptide immunotherapy for food allergy. Oral treatment with a peptide of ovomucoid significantly decreased allergic symptoms in a mouse model for egg allergy [113]. Intradermal treatment with a peptide of αS1 casein reduced T-cell and antibody responses to the intact protein in a cow's milk allergy mouse model [114]. In addition, treatment with partial whey hydrolysates reduced allergic symptoms also in this cow's milk mouse model [61, 62]. Interestingly, during the hydrolysis of whey proteins, peptides can be generated that are too small to induce basophil

activation but long enough to induce T-cell activation, but whether these peptides are still able to induce tolerance remains to be established [115].

In mice, oral treatment with peptides obtained by tryptic hydrolysis of β-lactoglobulin (β-LG), the major allergen in the whey fraction of cow's milk, reduced the allergic responses to intact β-LG [116]. Also, partial whey hydrolysates diminished the allergic symptoms in a mouse model for CMA [117]. However, treatment with extensive whey hydrolysates, which contained only small protein fragments (<5 kDa), had no effect. Similar results were seen in a study with rats [63]. Although tolerance could be induced using partial whey hydrolysates, it remains to be elucidated which exact fragments are responsible for the observed effects.

Peptide Therapy for Peanut Allergy

In food-allergic patients, oral tolerance to food proteins is disrupted. For years, it was thought that decreasing the exposure to allergens would reduce the development of allergy [22, 24, 25, 69]. Therefore, children at high risk of developing food allergy have been advised to eliminate allergens from their diet. However, recent studies have indicated that exposure to allergens may be beneficial [118, 119]. In Jewish children in Israel, in which the common practice is to consume peanut snacks at an early age, a lower risk of developing peanut allergy was observed compared to Jewish children in the UK who were not exposed to peanut [74]. Interestingly, not only entire proteins but also protein fragments may be used to induce oral tolerance [120].

The identification of the dominant T-cell epitopes of the major peanut allergens is crucial for the development of vaccines for peanut allergy. The dominant T-cell epitopes of *Arachis hypogaea* (Ara h) 2 and Ara h 1 are revealed and provide the potential use of these peptides to treat peanut allergy in humans [27]. Initially, for Ara h 2, two highly immunogenic T-cell-reactive regions were identified, Ara h 2 (amino acids 19–47) and Ara h 2 (amino acids 73–119), and peptides spanning these two regions induced strong T-cell proliferation associated with a Th2-type cytokine response [121]. Knowledge of the dominant T-cell epitopes of allergens is critical information for the development of a T-cell targeted vaccine for peanut-specific allergen immunotherapy. These short immunotherapeutic-peptides mitigated the significant anaphylactic potential associated with a low-dose administration with whole peanut proteins to peanut allergic patients, while theoretically maintaining the ability to induce a tolerizing immune response.

Meanwhile, 5 dominant Ara h 2 and 10 dominant Ara h 1 T-cell epitopes were identified that were not recognized by IgE from peanut-allergic patients [122, 123]. These T-cell epitope peptides could be presented by various HLA class II types (HLA-DR, HLA-DP, and HLA-DQ), showing their potential as candidate peptides for T-cell-targeted peptide immunotherapy in peanut allergy [98].

Potential Immunological Mechanisms Underlying Tolerance Induction by Successful Peptide Vaccine Treatment

Peptide vaccine treatment favors tolerance induction to specific allergens by altering T-cell polarization in allergic individuals. This peptide-based type of AIT alters T-cell polarization by altering direct antigen-presenting cell (APC)-T-cell interactions and cytokine secretion patterns (indirect APC-T-cell interactions) that are established during allergen exposure [99–101, 124].

Immunological Mechanisms of Tolerance Induction

The potential immunological mechanisms underlying tolerance induction by successful peptide vaccine treatment are yet to be fully defined but probably include similar changes in allergen-specific T-cell responses as those occurring during conventional AIT [23, 79, 125, 126]. Altering direct and indirect APC-T-cell interactions play a

major role in tolerance induction [99–101, 124] and comprise immune deviation, anergy of allergen-specific Th2 cells, deletion of allergen-specific Th2 cells, and active suppression of allergen-induced immune activation by Treg cell induction [23, 104, 127].

Immune Deviation

In conventional and T-cell-targeted AIT trials, a shift occurs in allergen-specific Th2 to Th1 cells at sites of allergen exposure during AIT [118, 119, 128, 129] with an increase in the Th1/Th2 cytokine ratio, leading to higher levels of interferon (IFN)-γ and lower levels of interleukin (IL)-4 and IL-13 [118, 119, 129]. This shift in cytokine responses during AIT, which is termed "immune deviation,", is responsible for the induction of tolerance and changes observed in allergen-specific antibody production [130]. As a result, a decline occurs in allergen-specific IgE levels, while allergen-specific IgG4 levels often increase. IgG4 competes with IgE for allergen binding to mast cells, basophils, and other cells expressing high-affinity IgE receptors, thereby blocking the ability of IgE antibodies to induce inflammatory mediator release by mast cells and basophils [6, 81, 131]. These IgG antibodies bind IgG4-allergen complexes to inhibitory receptors on mast cells to trigger deactivating signaling cascades [18, 81, 95]. Remaining IgE antibodies can no longer exert their adverse effects, resulting in reduced skin prick test responses in individuals successfully treated by AIT, indicating the suppression of basophil activation and mast cell reactivity [76, 132]. Also, the number of basophilic granulocytes and mast cells at sites of allergen exposure is diminished [133]. Mechanistic evidence for allergen-specific IgG production, and for the role of these antibodies in blocking effects of IgE antibodies, is less convincing for peptide therapy than for conventional AIT with whole allergen extracts which might be due to the presence of peptides too short to cross-link mast cell- and basophil-bound IgE receptors [80].

Anergy and Deletion of Allergen-Specific Th2 Cells

Proper T-cell activation requires antigen recognition, costimulatory receptors, and cytokine secretion. In the absence of one of these three key requirements, APC-T-cell interactions result in anergy of the specific T cell [13], reflecting a state of unresponsiveness, which is accompanied by impaired proliferation and production of cytokines upon allergen encounter [134]. The allergenic peptides present in T-cell epitope-based peptide vaccines are considered to be able to induce anergy of their matching allergen-specific T cells probably based on an intracellular signal transduction blockade, the expression of inhibitory receptors, and/or a lack of proper costimulatory signals [6, 93]. This results in a reduced expression of the T-cell receptor (TCR) and reduced TCR-CD3 receptor interaction. Since T-cell function of anergic T cells was not restored upon recovered TCR expression, it was indicated that anergy was not due to the reduced expression of the TCR but rather the induction and maintenance of defects in the intracellular signaling cascade [94]. This defect can occur in the activity of lymphocyte-specific protein tyrosine kinase (Lck) and ζ-associated protein of 70 kD (ZAP-70) kinases, as shown in peptide therapy for bee venom allergy [94].

Anergy induction can also occur via an increased expression of inhibitory receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), on T cells, as well as via a lack of proper costimulatory signals. CTLA-4 is expected to mediate its immunosuppressive function by interaction with CD80/CD86, leading to activation of phosphatases that dephosphorylate, and thereby deactivate, CD28-associated signaling molecules. Alternatively, the availability of CD80/CD86 for binding to CD28 can be reduced by capturing and endocytosing these APC-derived receptor molecules with a higher affinity than CD28 resulting ultimately in anergy [6, 135].

The ability of T-cell epitope-based peptide vaccines to induce anergy in allergen-specific T

cells of humans was shown by several in vitro studies [80, 87, 103, 111, 136–138]. Incubating cloned T cells specific for house dust mite (HDM) with an excess of their HDM allergen-specific dominant T-cell epitope peptides resulted in T cells presenting allergen peptides to each other as non-professional APC [20, 136, 138]. When these T cells were challenged by subsequent exposure to HDM allergen, only a short-term increased release of IL-4 was observed after which these T cells became anergic, characterized by suppressed proliferation [79]. Addition of exogenous IL-2 was shown to reestablish the proliferation and cytokine production of these T cells. The possibility of restimulation suggests that (in this case) the suppressed proliferation of these T cells was due to anergy rather than to deletion and resulted in tolerance [136].

Active Suppression of Allergen-Induced Immune Activation

The induction of Treg cells is considered to be one of the most important underlying mechanisms by which peptide-based immunotherapy may induce tolerance [23]. Treg cells actively inhibit the allergen-mediated immune activation that normally causes the symptoms observed in food-allergic individuals upon allergen exposure by immunoregulatory cytokine production, altering APC-T-cell interactions, and competing with other Th cell subsets for the growth factor IL-2 [6].

The transforming growth factor (TGF)-β produced by Treg cells is known to have several immunosuppressive and immunoregulatory functions, as it inhibits both Th1 and Th2 cell proliferation and cytokine production. In addition, TGF-β is suggested to promote tissue repair after allergic inflammatory responses. Furthermore, this cytokine enhances forkhead box protein 3 (FOXP3) expression, which is a transcription factor involved in the development and function of Treg cells [139]. CD103+ DCs in the gut-associated lymphoid tissue (GALT) promote FOXP3 expression via the secretion of TGF-β and retinoic acid thereby favoring Treg cell generation upon antigen presentation to naïve T helper (Th)0 cells [140]. Syed et al. (2014) indicated that immunological tolerance induction by successful OIT is associated with demethylation of the promoter region of the gene encoding FOXP3, thereby enhancing FOXP3 expression that stimulates the development and function of Treg cells [141]. Moreover, in an AIT trial with grass pollen allergen, it was found that allergic individuals had higher levels of FOXP3-expressing Treg cells in their nasal mucosa following successful AIT [18]. These findings indicate an important role for TGF-β secretion in tolerance induction by Treg cells during successful AIT, and likely also in T-cell epitope-based peptide immunotherapy [23, 79, 125].

Next to TGF-β, also IL-10 is known to have several immunosuppressive functions. IL-10 is considered to induce the anergic state of Th2 cells by inhibiting expression of costimulatory receptors and major histocompatibility complex (MHC)-II molecules on APCs [13, 134]. In the presence of IL-10, DC cannot fully differentiate as shown by a decrease in the expression of MHC-II molecules and CD80/CD86 costimulatory receptors [142]. Therefore, maturing DCs exposed to IL-10 in vitro are able to inhibit T-cell activation and cytokine production of both the Th1 and Th2 cell types [143], and this inhibition was found to be long-lasting since cytokine secretion of these T cells remained low, even when reexposed to regular mature DCs. When the T-cell growth factor IL-2 was added to the microenvironment of these T cells, these T cells could be reactivated again. This finding suggests that the immunosuppressive effect of these DCs exposed to IL-10 was due to anergy rather than to deletion [143]. Moreover, IL-10 is suggested to downregulate FcεRI expression on mast cells and basophils, which indicates another immunosuppressive function of this cytokine in allergic manifestations [18, 81].

Treg cells inhibit the capacity of APCs to induce T-cell stimulation not only by the production of IL-10 and TGF-β, but also by altering direct APC-T-cell interactions. Such an altered

direct APC-T-cell interaction is the inhibition of CD28-CD80/CD86 interaction by CTLA-4 expression on Treg cells [6, 135]. The expression of CTLA-4 by Treg cells is not considered to be the only important factor for the immunoregulatory role of these cells. Also, the interaction between inducible T-cell costimulator (ICOS) on Treg cells and its ligand (ICOSL) on DCs is expected to be crucial for the immunosuppressive function and IL-10-producing capacity of these Treg cells [144]. Treg cells also inhibit the proliferation and differentiation of other Th cell subsets by competing for the single most important T-cell growth factor IL-2. Since Treg cells express constitutively high levels of the IL-2 receptor (CD25), they may deprive other Th cell subsets from this growth factor necessary for proliferation, growth, and survival of these Th cells [101, 145].

T-Cell Epitope-Based Peptide Therapy Activates Tolerance-Inducing Mechanisms

T-cell epitope-based peptide vaccine treatment is considered to favor tolerance induction by its characteristic physical form, its tolerance-favoring route of administration, and high-dose allergen exposure [81, 146]. The physical form of the allergen determines the possible cell-cell interactions and release of cytokines [81]. While native allergen molecules can cross-link APC-bound allergen-specific antibodies, short T-cell epitope peptides present in peptide vaccines cannot. As a consequence, these short synthetic peptides are not able to fully activate APCs, leading to a quiescent interaction between APCs and T cells with no inflammatory T-cell responses resulting in the generation of Treg cells and induction of immunological tolerance [80, 147].

Peptide vaccine trials initially used the subcutaneous route of administration, but subsequent trials have focused on an intradermal route of administration, which has a higher immunogenicity, tolerability, and (due to easy administration) a higher practical applicability. This shift improved both treatment efficacy and patient compliance [96, 137]. Also, intradermal administration of epitope-based vaccines to the noninflamed skin is safer and more effective than conventional AIT with whole allergen extracts due to the systemic presentation of allergenic peptides [126]. These peptides are presented by MHC-II-expressing nonprofessional APCs in the noninflamed tissues, like endothelial cells, epithelial cells, and immature DCs to naïve T cells. These nonprofessional APCs, which lack proper costimulatory receptors, inhibit T-cell activation and induce differentiation into Treg cells rather than Th2 cells [81, 125, 147, 148].

T-cell epitope-based peptide vaccines expose patients to higher doses of allergen compared to natural low-dose allergen exposure [111, 120] resulting in preferential Th1 cell induction by increasing the expression of CD40L on Th cells facilitating interaction with CD40, which is constitutively expressed on APCs. This interaction induces the secretion of IL-12 driving this Th1 cell polarization. Low-dose allergen exposure, on the other hand, favors IL-4-induced Th2 cell development, for it is unable to trigger CD40L expression resulting in low IL-12 secretion. However, the interaction between leukocyte function-associated antigen 1 (LFA-1) on Th cells and intracellular adhesion molecule 1 (ICAM-1) on DC interferes with this T-cell polarization effect. LFA-1-ICAM interaction can reduce the allergen dose required for increased CD40L expression. Although the exact mechanisms remain to be elucidated, high-dose allergen exposure was also shown to induce Treg cell development and tolerance induction [127].

Clinical Relevance of T-Cell Epitope-Based Peptide Vaccines

When cat-allergic individuals were subcutaneously injected with short peptide fragments consisting of dominant T-cell epitopes of cat allergen (Felis domesticus 1, Fel d 1), they had significantly fewer upper and lower airway problems when exposed to cats compared to placebo-treated cat-allergic individuals [103]. Since the adverse responses accompanying this treatment

were shown to be mild and few, it was concluded that the patients tolerated the injections seemingly well [103]. A subsequent trial showed that when higher amounts of even more different cat peptides were included in the vaccine, the treatment efficacy was significantly increased [111]. In both trials, an associated increase in IL-10 production was observed, together with a decrease in Th2 responses [84]. Bee venom-allergic individuals were treated with T-cell epitope-based peptide vaccines containing the dominant T-cell epitopes of phospholipase A2, which is the major bee venom allergen [109]. When these individuals were subsequently exposed to phospholipase A2, their allergic manifestations were significantly reduced. At the same time, the proliferation of allergen-specific Th cells was found to be reduced, indicating the anergic state of these Th cells reflecting successful tolerance induction [109].

Few studies have focused on the potency of peptide vaccines in peanut allergy or food allergy in general [76]. In a clinical study with mice, epicutaneous sensitization was followed by intraperitoneal treatment with T-cell epitope-based peptide vaccines containing peptides comprising only one specific Ara h 1 T-cell epitope, once after 7 days and again after 14 days. Six different doses were included, ranging from 0.01 μg to 300 μg of Ara h 1 peptide. After treatment, these mice were challenged by intraperitoneal injection with whole peanut extract after which occurrence of anaphylaxis was evaluated by monitoring them over a period of 40 minutes. A significant decrease in anaphylaxis was observed in the treatment group compared to the peanut-sensitized but saline-treated control group, following a dose-dependent pattern. The amount of Ara h 1-specific T cells was found to be significantly lower in tissue samples of the treatment group compared to the control group. Treatment with a vaccine that contains only one type of Ara h 1 T-cell epitope may thus already reduce the risk of anaphylaxis after a challenge with whole peanut extract [149, 150]. In a different study with mice, focusing on epitopes of Ara h 2, a peptide vaccine comprising 30 overlapping Ara h 2 peptides of 20 amino acids long was subcutaneously administered. This vaccine treatment significantly lowered levels of histamine and Ara h 2-specific IgE antibodies, and reduced clinical symptoms of anaphylaxis after peanut allergen challenge [151]. The results from these studies illustrate that the potency of T-cell epitope-based peptide vaccines in peanut allergy treatment is expected to be high [6, 27, 149–151].

Critical Appraisal

Despite the clinical potency of T-cell epitope-based peptide vaccines, only a few studies have been published concerning T-cell epitope-based peptide vaccines in relation to peanut allergy or food allergy in general [27, 79]. Nevertheless, results from trials evaluating the potency of peptide immunotherapy in allergy treatment to aeroallergens are highly encouraging as T-cell epitope-based peptide vaccines appear to be a safe and effective new class of vaccines for allergy treatment, potentially enabling the wider application for more severe allergies (such as peanut allergy) [73]. The incorporation of peanut allergen-specific peptide vaccines in clinical practice is dependent on the identification of the dominant T-cell epitopes within the two major peanut allergens (Ara h 1 and Ara h 2) and the optimal composition and dosing interval of a peanut allergen-specific peptide vaccine. Next, it is important to test whether the risk of potential allergic responses during treatment is indeed negligible, ensuring the safety of the treatment [134]. After all, it is highly relevant to consider the practical applicability of peptide vaccines in peanut allergy treatment when speculating about the potency of this treatment.

For application of a T-cell epitope-based peptide vaccine for peanut allergy treatment, knowledge about the immunodominance T-cell epitopes in Ara h 1 and Ara h 2 is needed since the strongest immunogens are also considered to be best able to induce tolerance [23]. Immunodominant epitopes are those epitopes that have the highest affinity to bind to MHC molecules [6]. These epitopes can only be identified when the amino acid sequences of peanut allergens are known, and

isolation and identification of peanut allergen-specific T cells from peanut-allergic individuals have been performed. However, it is important to investigate whether these epitopes, identified by in vitro studies, actually reflect those of significance in vivo [134]. Immunodominant T-cell epitopes of aeroallergens found in in vitro studies proved to be of great predictive value for the relevant epitopes in vivo [89, 152]. Despite the fact that these results are promising, it remains to be investigated whether results from in vitro approaches used for identification of dominant peanut T-cell epitopes actually reflect the immunodominant epitopes in vivo [134].

The optimal composition and dosing schedule of a peanut allergen-specific peptide vaccine for tolerance induction in allergic individuals needs to be established. The optimal dose for tolerance induction may differ between different types of allergies [79]. It is still not completely clear whether high-dose allergen exposure is necessarily better than low-dose allergen exposure in establishing sustained unresponsiveness [77]. In addition, the optimal peptide length and number of peptides present in the peptide vaccine remain to be evaluated [148, 153]. Longer peptides often comprise more T-cell epitopes, thereby increasing treatment efficacy, but they also increase the risk of cross-linking mast cell- and basophil-bound IgE antibodies, thereby inducing adverse allergic responses [29]. Therefore, vaccines containing a larger number of short peptides may provide a better option than vaccines containing a smaller number of long peptides. However, many different peptides increase the complexity of the vaccine, and this may cause problems with the solubility and stability of the peptides in the vaccine since it enlarges the possibility of interactions between peptides. These interactions between peptides may facilitate peptide aggregation and thereby the formation of large peptide complexes [103]. Therefore, some peptides require certain minor modifications in their structure, like individual amino acid substitutions to improve their solubility and stability before they can be used in vaccines [79, 154]. A vaccine comprising all possible T-cell epitopes is not yet feasible and deliberate choices should be made regarding which epitopes are most important to include in the vaccine [154].

However, it is hard to determine which epitopes are most important to be included in the vaccine due to the high variation in epitope recognition between peanut-allergic individuals (interindividual differences due to different MHC class II molecules) and sometimes also within a peanut-allergic individual (intraindividual differences). The presence of intraindividual differences was confirmed as the degree of epitope diversity was higher for allergic subjects with a history of peanut-induced anaphylaxis than for those only experiencing mild adverse responses upon peanut exposure [155]. These findings underscore why peptide vaccines should be able to suppress T-cell reactivity to multiple epitopes at the same time, especially in people suffering from these severe peanut-induced systemic responses. However, treatment with a peptide vaccine containing only one dominant epitope of the cat allergen Fel d 1 did not only inhibit the allergic T-cell response to this epitope but to the whole Fel d 1 allergen [156]. This phenomenon, known as linked epitope suppression, may indicate that although different patients have different sensitization patterns, treating them with a vaccine containing one specific epitope of an allergen may lead to desensitization to the whole allergen [157]. This phenomenon would explain how a small number of allergen-specific dominant T-cell epitopes may induce tolerance to all epitopes in that allergen [20]. Therefore, linked epitope suppression induced by peptide therapy indicates that peptide vaccines may be more effective in inducing tolerance than initially expected [158]. However, the relatively complex nature of peanut allergy compared to cat allergen does not necessarily prove that linked epitope suppression occurs during peptide vaccine treatment with peanut allergens [21].

Although all the candidate peptides in the peptide vaccine are considered too short to cross-link cell-bound IgE, it is crucial to demonstrate that these peptides are indeed not able to induce mast cell and basophil degranulation. To test this, the

basophil activation tests (BAT) should be performed with blood samples from peanut-allergic individuals in the presence of the peptides from the peptide vaccine, singly or in combination. This should be tested for different peptide vaccine concentrations in order to determine the optimal therapeutic dose [29]. Basophil activation can be assessed by determining histamine release and/or by determining the presence of the coreceptor CD63 on mast cells or basophils by flow cytometry [79, 82].

Increased efficacy of conventional AIT can be achieved when combined with a clinical grade anti-IgE antibody (Omalizumab) as this combination was shown to reduce adverse allergic responses to conventional AIT [24]. However, for most conventional AIT-treated peanut-allergic individuals, transient desensitization still appears to be the highest feasible at present [29].

Conclusions

Specific immunotherapy of sensitized patients with vaccines is most advanced for respiratory allergies, and this progress can help to advance the development of defined vaccines for food allergy. There is also a need for further clinical trials in order to confirm whether promising in vitro effects of T-cell epitope-based immunotherapy are indeed translated to clinical efficacy and safety [134]. Although T-cell-targeted immunotherapy may really have potential for treating cow's milk and peanut-allergic individuals in the future, strict avoidance of milk or peanuts and food containing milk or peanuts remains the standard approach for food allergy management for now [27, 29], but we anticipate profound advances in the treatment of food allergies through allergen-based vaccines in the near future.

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Obesity and Immunity

18

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Key Points

- Diet-induced obesity can increase the amount and function of adipose tissue via the production of metabolically active cytokines and hormones especially in the white adipose tissue and skeletal muscle through bidirectional cross talk between these two organs.
- Under conditions of excessive caloric intake, adipose tissue undergoes different immune changes such as migration and activation of macrophages, natural killer cells, and lymphocytes and production of pro-inflammatory cytokines.
- Loss of immune inhibitory mechanisms results in tissue inflammation contributing to obesity and related metabolic complications.

Introduction

Obesity is reckoned as a fast-growing problem resulting from fat accumulation. It is related to numerous chronic autoimmune and chronic inflammatory diseases, particularly cardiovascular disorders and type 2 diabetes mellitus. It also contributes to increased mortality rates from all causes [1–4]. In 2016, more than 340 million children and adolescents were overweight or obese and 650 million adults were obese. Since 1975, the prevalence of obesity has nearly tripled in all over the world [5]. World Health Organization (WHO) has proposed body mass index (BMI), calculated as a ratio of weight in kilograms to the square of the height in meters, for the classification of overweight and obesity in adults. BMI of 25–29.9 kg/m^2, 30.0–34.9 kg/m^2, 35.0–39.9 kg/m^2, and 40 or greater kg/m^2 represents the classification of overweight and grade I, II, or III obesity, respectively [6]. Many epidemiological studies support the hypothesis that obesity affects the immune function [6, 7]. For instance, clinical evidence indicates the higher incidence of specific infections in obese people compared with lean people [8, 9]. Also, obesity is associated with the development of secondary infections and complications such as sepsis, bacteremia, and poor antibody responses to vaccine [10].

Adipose Tissue: Types and Functions

For a long time, scientists focused on the identification of different risk factors and treatments for obesity. It seems that strategies concentrating on diet, physical activity, and pharmacotherapy have not been effective [11]. Adipose tissue (AT) is an important organ in that it stores excess calories and regulates energy mobilization by circulating lipid according to energy status [9, 11]. In diet-induced obesity (DIO), adipocytes undergo hypertrophy resulting in the expansion of the visceral AT (vAT). This condition is a strong predictor of insulin resistance (IR) [12]. In obesity-induced low-grade inflammation, several kinds of pro-inflammatory immune cells including monocytes, macrophages, natural killer cells, and lymphocytes may infiltrate into the AT, resulting in the secretion of adipocytokines by both adipocytes and infiltrated immune cells [12, 13].

White Adipose Tissue

White adipose tissue (WAT) is the most important organ for lipid storage and WATs are distributed throughout the body, particularly muscle, skin (subcutaneous WAT), and surrounding internal organs (visceral WAT) [14]. The most of the total body fat (about 80%) is concentrated in subcutaneous (sc) WAT though visceral fat accounts for about 5–8% of total body fat in women and 10–20% in men [7]. The parenchymal cells of WAT, white adipocytes, contain a large lipid droplet surrounded by a thin layer of cytoplasm and a peripheral flattened nucleus, leading to a typical signet ring appearance [14]. The main function of WAT is to store nutrients and energy in form of triacylglycerol (TAG) by taking up lipids from the blood circulation and accumulating them in cytoplasmic lipid droplets (LDs) within adipocytes (fat cell) [11, 15]. When excess calories are consumed, white adipocytes provide fuel

for other organs by releasing free fatty acids from the lipid droplets [7]. Increased lipolysis by sympathetic stimulation is mediated by norepinephrine binding to beta-adrenergic receptors, which would initiate the production of intracellular cyclic AMP, the second messenger, resulting in activation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [14]. Besides its role in the energy storage, WAT has been recognized as a complex organ which secretes adipocytokines and metabolites with endocrine, paracrine, and autocrine functions mainly related to the energy and glucose homeostasis [16, 17]. It can secrete hormones and cytokines such as leptin, adiponectin, and tumor necrosis factor-alpha (TNF-α) and many other interleukins.

Brown Adipose Tissue

Brown adipose tissue (BAT) has a crucial role in heat generation. The amount of BAT drops after infancy and childhood. However, BAT remains functionally active in adults. Unlike WAT, BAT squanders energy to reduce body mass and restore lipid and glucose homeostasis [17]. Studies of human adults have shown that the activity of BAT in obese and overweight adults is low and persons with larger BAT mass have a higher resting metabolic rate (RMR) [14, 18, 19]. BAT adipocytes contain many small lipid droplets (multilocular) which is different from white adipocyte with a large lipid droplet (unilocular). Furthermore, brown adipocytes are rich in mitochondria carrying a special proton transporter at the inner membrane, which is capable of transferring energy as heat by stimulation of β-adrenergic receptors [14, 17].

Beige Adipose Tissue

More recently, other adipocytes which are morphologically similar to white adipocytes but functionally similar to brown adipocytes are studied. These cells are generally referred to as "beige" or "brite" (for brown-in-white) adipose cells. It seems that these cells like brown fat cells are crucial in the regulation of basal metabolic rate (BMR) and obesity [19, 20].

Adipose Tissue and Immune Cell

Immune cells have important roles in health and disease. One of the most important functions of the immune system is inhibition of tissue inflammation. Loss of immune inhibitory mechanisms causes the tissue inflammation contributing to either reduced or increased AT mass and thereby affecting different functions of this tissue [14, 21]. Obesity is associated with local and systemic inflammation thought to originate predominantly from AT [6, 22].

Macrophages

Despite the importance of all immune cells in health and disease, macrophages are of particular importance because they are found in all metabolic tissues [23]. Tissue-resident macrophages are responsible for the development and maintenance of tissue homeostasis, and their names have been given based on their tissue-intrinsic function [24, 25]. In a traditional classification, macrophages in AT are categorized as M1- like or M2-like. M1-like macrophages are considered to be pro-inflammatory and secrete cytokines interleukin (IL)-6, TNF-α, and IL-1β and chemokines such as chemokine (C-C motif) ligands 2, 3, and 5 (CCL2, CCL3, and CCL5). M2-like macrophages are induced by transforming growth factor β (TGF-β), IL-4, and IL-13, and they are generally anti-inflammatory and profibrogenic thus protecting against the chronic inflammation which is the characteristic of unhealthy AT [23, 25]. New studies have identified another type of macrophages, named metabolically active macrophages (MMes). MMes secrete pro-inflammatory cytokines, but they have different cell surface markers which are different from both M1 and M2 macrophages [26]. For instance, ABCA1, CD36, and PLIN2 are cell-surface markers expressed by MMes that play role in lipid metabolism. On the other hand, M1-like macrophages express CD38, CD319, and CD273, which are known as traditional M1 markers. In addition, macrophages with iron-handling capacity, which were first identified in atherosclerotic plaques, have been found in AT. These macrophages have unique phenotypes based on

haptoglobin-hemoglobin complex uptake M (Hb) or hem uptake (Mhem) and display an M2-like phenotype [27]. Recent study has found another type of iron-handling macrophage in AT which is called MFe^{hi} and characterized with M2-like phenotype high expression of CD163. These macrophages have more iron content and iron-handling genes than non-iron-handling AT macrophages [25]. With obesity, M2-type macrophages shifted to M1-type macrophages which may be influenced by IL-6 or TNF-α. Mitogen-activated protein kinase (MAPK) is a key pathway in macrophage-mediated inflammatory responses and may play a significant role in diseases mediated by macrophages [23].

Mast Cells

In adipose tissue, mast cells are found between adipocytes next to microvessels and can contribute to obesity [6, 28]. These cells have crucial role in the development of chronic low-grade inflammation in adipose tissue by their potential influence on immune cell attraction and activation especially in vAT compared to scAT [28, 29]. At molecular level, mast cell degranulation can stimulate the secretion of pro-inflammatory cytokines, chemokines, and pathways. Additionally, mast cells modulate AT remodeling and fibrosis by affecting adipocyte differentiation and fibroblast proliferation as well as enhancing the expression of extracellular matrix proteins [29, 30]. Further, mast cells are responsible for the regulation of insulin resistance by their histamine reserve. Histamine is a biogenic amine inflammatory mediator. In the form of the histamine-forming enzyme histidine decarboxylase (HDC), it has a critical role in mast cell function and in provoking insulin resistance. HDC is responsible for the production of histamine from histidine. Previous studies suggested HDC as a contributing factor to the inflammatory processes and therefore the development of T2DM [31, 32].

Neutrophils

An early transient neutrophil infiltration is one of the most important characteristics of classic innate immune responses including inflammation [32]. This reaction is characterized by phagocytosis and migration of neutrophils to the infected site, leading to the secretion of cytokines and chemokines such as TNF-α, IL-8, IL-1β, and macrophage inflammatory protein-1 alpha (MIP-1α). The mechanism of neutrophil activation in obesity is incompletely understood. Evidence shows that neutrophil counts in obese female adolescents are associated with BMI, waist circumference (WC), and total AT [28]. Inhibition of neutrophil activation by affecting macrophage infiltration into AT improved obesity-induced IR as well as suppressed inflammation in metabolic organs such as AT and liver. Interaction between CD11b on neutrophils and intercellular adhesion molecule 1 (ICAM-1) from adipocytes mediates adhesion between adipocytes and neutrophils [33]. A high-fat diet (HFD) causes significant increase in neutrophil number up to 20-fold after 3 days. Neutrophil accumulation mostly occurs in visceral fat, but not in subcutaneous fat [28, 32], and promotes elastase production which, in turn, upregulates the expression of cytokine genes. In this manner, neutrophil activation and accumulation would accelerate metabolic disorders like glucose intolerance, IR, non-alcoholic fatty liver disease (NAFLD), and atherosclerosis [7].

Eosinophils

There is evidence that eosinophils are present in AT and may regulate metabolism by improving insulin sensitivity and anti-inflammatory responses especially in the vAT [6, 34, 35]. Eosinophils are found in the AT in small amounts. Recent studies suggested that eosinophil numbers decline with DIO. This demonstrates the crucial role of eosinophils for the maintenance of normal metabolic function. The exact role of eosinophils in obesity has not been well-quantified. However, it has been shown that eosinophil-derived Th2 cytokines (TGF-β, IL-1, IL-4, and IL-13) maintain tissue-resident ATMs in an alternatively activated M2 state and stimulate both B and T cells to counter harmful effects of obesity [32]. A HFD decreases eosinophil numbers in AT. A recent study has shown that

obese mice lacking eosinophil exhibit greater degrees of IR [6]. Eotaxin is an 8.3 kDa chemokine which acts on eosinophils. Elevated levels of eotaxin have been detected in different metabolic conditions ranging from coronary heart disease to obesity [36]. Obese persons have high levels of eotaxin in AT and serum. This indicates that obesity is accompanied by activation of allergic innate immune responses [28].

Dendritic Cells

Dendritic cells (DCs) are key participants in both the innate and adaptive immunities by presenting antigens to T cell receptors [32, 37]. DCs are the primary antigen-presenting cells (APCs) yet secrete various cytokines, e.g., IL-12 and IL-15, that trigger the adaptive immune cells. Although CD80, CD83, and CD86 are also known as markers for DCs, CD11c is the main surface marker for DCs [32]. DCs are a heterogeneous population of cells including the conventional/myeloid DCs (cDCs) and plasmacytoid DCs (pDCs). Both of them were first separated from the spleen but have been subsequently found in most tissues. Recently, a novel type of DCs known as inflammatory DCs (inf-DCs) was introduced. As the name implies, inf-DCs are generated from inflammatory monocytes and related to infection or inflammation [37, 38]. A HFD causes accumulation of dendritic cells in AT. Therefore, the expression of DC antigens in obese cases is increased compared to lean counterparts via. Further, a HFD induces DCs to secrete some chemokines and cytokines required for the recruitment of immune cells, Th17 cell differentiation, and M1 polarization. Moreover, the number of CD11c cells is correlated with IR due to down-regulation of glucose transporter type 4(GLUT-4) transporter in adipocytes and blocking insulin signals by TNF-α [32].

B Cells

B cells are main contributors to humoral immunity. They produce antibodies specific for antigens through toll-like receptors (TLRs), and accordingly are divided into two main groups: B-1 cells and B-2 cells [32]. In particular, B-1 cells have a crucial role in the secretion of immunoglobulin following pathogen encounter. They are able to secrete natural IgM in germ-free mice and in the absence of immunization, which might be beneficial to prevent obesity [24]. B-2 cells are responsible for germinal center reaction and IgG production. Generally, both B-1 and B-2 cells contribute to the production of microbiota-specific IgA [24]. The exact role of B cells in obesity is not completely known, but recent studies have indicated that B cell infiltration correlates with obesity and IR. A HFD promotes M1 polarization, macrophage accumulation in vAT, and pro-inflammatory cytokine production through increasing B cell numbers [7, 32]. Further, during obesity, B cells decrease the production of IL-10 in AT, and in this manner a HFD can amplify inflammation. Recently, it has been shown that regulatory B cells (Bregs) which produce an anti-inflammatory cytokine (IL-10) have a regulatory effect on obesity-induced IR. This population of B cells seems to be more abundant in lean mice than obese mice [39].

T Cells

T cells are synthesized in the bone marrow and migrate to the thymus where they can be matured. They can be divided into two subtypes depending on their surface markers, CD4 and CD8 T cells. Also, depending on specific function, T cells are categorized into cytotoxic T cells, regulatory T cells, and helper T cells. Helper T cells express CD4 as are differentiated into Th1, Th2, Th17, and Treg based on the type of produced cytokines. Cytotoxic T cells which express CD8 at their surface produce perforins and some cytokines and thereby kill infected cells and cancer cells [32]. T cells play a crucial role in obesity-induced inflammation. The insulin receptor augments T cell activation to support glucose metabolism. Studies have shown that insulin promotes polarization toward a Th2 phenotype [40]. Obesity-induced alteration in insulin sensitivity leads to the recruitment of inflammatory cells into vAT. This seems to be mediated by increased regulatory T cells (Treg and Th2) and inflammatory (Th1 and CD8+) T Cells [41]. Recent studies

have indicated that DIO accelerates the accumulation of both $CD8^+$ and $CD4^+$ T cells in vAT, along with a shift to Th1/Th17 T cell numbers [42]. IFN-γ released from inflammatory T cells is responsible for macrophage polarization toward an M1 phenotype [40, 41, 43].

Adipocytokines and Obesity

Adipocytokines acting as special brain hormones regulate appetite and nutrient metabolism. Further, adipokines have a role in immunity and inflammation. Studies reveal the presence of a low-grade inflammatory state in people with overweight and obesity [3]. Additionally, different studies have demonstrated that DIO increases the amount and function of AT through the production of pro-inflammatory cytokines and hormones influencing metabolic function [44]. Supporting this, obesity confers an increased susceptibility to cardiometabolic disorders including T2DM. Among AT depots, vWAT appears to have the largest influence on obesity-related diseases. This might be due to differences in adipokine secretion [45]. The information about global expression of adipokines in AT is limited. However, recent studies have investigated the secretion pattern of peptides from human subcutaneous progenitor cells undergoing in vitro differentiation to fat cells [46, 47].

Leptin

Leptin is one of the most important cytokine-like hormones mainly produced by vAT and by other organs such as brain, SKM, and GI tract in low levels [3]. In physiological conditions, leptin levels are correlated with the amount of vAT. Inflammatory factors can modulate synthesis of leptin [3, 48], which has a pivotal role in weight balance by inhibiting the expression of orexigenic neuropeptides such as neuropeptide Y (NPY) and inducing the expression of anorexigenic factors such as cocaine-amphetamine-related transcript (CART) in the brain [49]. Impaired leptin signaling in the central nervous system (CNS), particularly the hypothalamus, results in leptin resistance which is the major risk factor for obesity. Biologic functions of leptin are mediated via its binding to the long-form leptin receptors (LepRb) expressed in the brain and peripheral tissues. In innate immunity, leptin enhances inflammatory responses through increasing the cytotoxicity of NK cells and the activation of granulocytes (neutrophils, basophils, and eosinophils), macrophages, and DCs [50]. Leptin by the expression of surface markers for an M2-like phenotype influences the phenotype of AT macrophages (ATM). It affects DCs maturation and migration as well. Moreover, leptin augments the production of TNF-α and IL-6 in monocytes and CC-chemokine ligands in macrophages. These cytokines mediate inflammatory actions of this hormone [51]. Recent evidence indicates that TLRs are key factors in regulating the innate immune responses through affecting AT, obesity-related inflammation, and leptin. Leptin leads to increased production of cytokines including IL-2, IL-12, and IFN-γ [3, 51, 52]. In the adaptive immune system, leptin suppresses the proliferation of Tregs and production of anti-inflammatory cytokine IL-4 in T cells [51]. On the other side, the proliferation of naïve T and B cells as well as Th17 cells is enhanced by leptin. In fact, leptin promotes polarization of Th cells toward a pro-inflammatory phenotype. Leptin takes part in many physiological functions, including bone metabolism, inflammation, and immune responses [3, 49]. The main consequence of a high caloric diet may be decreased energy expenditure. In this manner, leptin and its produced inflammatory cytokines endeavor to closely link nutrition, metabolism, and immune homeostasis [3].

Adiponectin

Adiponectin (also known as GBP28, apM1, Acrp30, or AdipoQ) is the most abundant plasma protein exclusively synthesized in AT. It exerts multiple protective effects against inflammation, obesity, IR, and CVD [53, 54]. The inflammatory effects of adiponectin are mediated by controlling the function of M1 and M2 macrophages. Adiponectin downregulates the expression of pro-inflammatory cytokines, such as TNF-α, MCP-1, and IL-6 while inducing the expression of anti-inflammatory M2 markers, such as Arg-1 and IL-10. Adiponectin receptors and their

downstream signaling pathways in monocytes/macrophages mediate the anti-inflammatory activity of adiponectin [3]. In contrast, there is evidence showing that adiponectin can produce pro-inflammatory effects under special conditions [53]. Interestingly, adiponectin can affect other innate immune cells leading to the suppression of activity of eosinophils, neutrophils, γδ T cells, NK cells, and DCs [3, 55]. In severe obesity, adiponectin levels are decreased, whereas weight loss increases adiponectin levels. It has been shown that adiponectin acts as an endogenous insulin sensitizer by increasing glucose uptake through its ability to augment fatty acid oxidation and to reduce gluconeogenesis in the liver. Adiponectin downregulates antigen-specific T cell proliferation and cytokine production [55].

TNF-α

Tumor necrosis factor-alpha (TNF-α) was the first pro-inflammatory adipokine known to be released from the obese AT. This clearly indicates the close correlation between obesity, inflammation, and T2DM. Studies have shown that the expression of TNF-α mRNA is increased in AT and the neutralization of this cytokine improved insulin sensitivity in obese people [51, 55, 56]. TNF-α is principally expressed in monocytes and macrophages, but it can be released from T cells, B cells, NK cells, and fibroblasts in special conditions as well [57]. The increased levels of this cytokine in obesity are due to the increased infiltration of M1 macrophages in AT [55]. TNF-α induces the production of other pro-inflammatory cytokines such as IL-6 and IL-1β and thereby resulting in lipolysis. Moreover, TNF-α reduces insulin sensitivity and adipogenesis through its receptors: TNF receptors 1 and 2 (TNFR1 and TNFR2) [58].

Interleukin-6 (IL-6)

IL-6 is a soluble cytokine promoting the B cell differentiation into antibody-producing cells. Researchers have reported high levels of IL-6 (~15-fold) in insulin-resistant individuals. IL-6 participates in insulin signaling through tissue-specific actions [55, 57, 59]. IR can happen as a result of (a) decreased phosphorylation of the insulin receptor substrate (IRS) and Akt or protein kinase B (PKB) and (b) decreased transcription of the IRS in hepatocytes and adipocytes [57]. Moreover, AT-derived IL-6 is mostly released from cells of the stromal vascular fraction. IL-6 can modulate leptin production in AT and SKM and affect vAT mass by altering the glucose uptake capacity and lipogenic/lipolytic factors [60].

Interleukin-18 (IL-18)

The cytokine IL-18 is a member of the IL-1 family expressed in different tissues favorably liver and AT (particularly vAT). It has been shown that IL-18 in AT is not synthesized by adipocytes; IL-18 is, however, known for having a crucial role in inflammation [61] as well as energy metabolism, through its interaction with receptors such as transmembrane IL-18 receptors (α and β) and subsequent activation of signaling pathways including nuclear factor κB (NF-κB), gene phosphatidylinositol-3 kinase (PI3K)/Akt, signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinases (MAPK), and c-Jun NH2-terminal kinase (JNK) [62]. Studies have shown that serum levels of IL-18 are increased in obesity and type 2 diabetes [61].

Chemerin

Chemerin is a chemoattractant molecule for immature DCs and macrophages through the expression of several G protein-coupled receptors (GPCRs) including chemokine-like receptor 1 (CMKLR1) and chemerin receptor 23 (ChemR23) [63, 64]. It is considered an adipocytokine playing role in adipogenesis, AT metabolism, inflammation-related obesity, and IR [55, 57, 63]. Furthermore, serum chemerin levels are elevated in severe obesity. Decreased levels of chemerin following surgical weight loss correlate with an improvement in insulin sensitivity and blood glucose [16]. Chemerin produced by periaortic vascular adipose tissue (PvAT) would boost vascular contraction through immune cell-related receptors. But, there is a lack of evidence linking spe-

cific chemerin signaling pathways to the PvAT inflammation. However, researches have revealed that pro-inflammatory cytokines such as TNF-α, IL-1b, and IL-6 can upregulate the expression of chemerin receptor [65]. Accordingly, serum levels of chemerin are increased in chronic inflammation-related diseases such as obesity and metabolic syndrome [64].

Resistin

Resistin, also called FIZZ3 (found in inflammatory zone) or ADSF (adipocyte-specific secretory factor), is an adipocyte-derived hormone which was discovered in 2001 [66]. As the name implies, resistin plays role as a mediator of inflammation and IR by inducing the production of TNFα, IL-12, IL-6, and MCP-1 from various cell types including blood mononuclear cell (PBMCs) and pancreatic acinar cells [63]. On the other hand, TNF-α administration would decrease the mRNA expression of resistin in adipocytes and preadipocytes. The most studied biological effect of resistin is the regulation of glucose homeostasis and insulin sensitivity. It has been shown that obesity-related impairment in insulin sensitivity is accompanied by downregulated mRNA expression of resistin in AT. Interestingly, evidence suggests that resistin might be effective in controlling food intake due to its influence on the expression and activation of hypothalamic neurons. Animal studies have shown that the potential mechanisms of the short-term satiety effect of resistin could be involved: first, inhibition of mRNA expression of orexigenic neuropeptides, NPY, and agouti-related peptide (AgRP); second, induction of mRNA expression of anorexigenic CART; and third, blockade of brain-related fatty acid metabolism [66, 67].

Visfatin

Visfatin (also called NAMPT) is named "an adipokine-enzyme" predominantly secreted by lymphocytes of vAT. The functional role of visfatin is not completely understood, but it has been shown to have glucose-lowering and insulin-mimicking/insulin-sensitizing effects via binding to insulin receptor [66]. Also, visfatin appears to be an inflammatory mediator that acts to produce pro-inflammatory adipokines (IL-1β, TNF-α, and IL-6) as well as anti-inflammatory cytokines (IL-10 and IL-1). The potential mechanisms through which visfatin may promote atherosclerosis include (a) atherosclerotic plaque destabilization, (b) deterioration of cellular resistance to genotoxic/oxidative stress, (c) adhesion of leukocytes to endothelial cells, and (d) production of cytokines (IL-6 and IL-8) [66].

Apelin

Apelin is an endogenous bioactive peptide which belongs to the family of adipokines. It is produced by adipocytes, vascular stromal cells, and the cardiovascular system [57]. Apelin is able to act as the endogenous ligand of the G protein-coupled receptor APJ [68, 69]. Both apelin and APJ receptor are found in different tissues such as heart, brain, limbs, retina, and liver. Therefore, they can play role in the various physiological processes including blood pressure regulation, body fluid homeostasis, endocrine stress response, cardiac contractility, and energy metabolism [68]. Impaired apelin signaling has been implicated in cardiovascular diseases, obesity, and cancer [57]. Interestingly, apelin can regulate insulin sensitivity by influencing adiponectin levels and adiposity. Adiponectin, in turn, would decrease free fatty acid levels [57]. In this manner, apelin serves as a cardioprotective cytokine through modulation of the renin-angiotensin-aldosterone system (RAAS) [70].

Omentin

Omentin, also known as intelectin, is a lectin-binding protein highly expressed in vAT especially in women [63, 71]. Omentin-1 is an important form of omentin in serum. However, there are other homologs such as omentin-2. Genes of both forms are located in the chromosomal region linked to type 2 diabetes [72]. Omentin is found in the lung, intestine, heart,

ovaries, and placentas. It is known as an anti-inflammatory adipokine by suppressing the activity of TNF-α, CRP, and IL-6. Moreover, it can increase insulin signal transduction and glucose transportation with no effect on basal glucose uptake [73]. A recent study showed that omentin contributes to the regulation of lipid metabolism by suppressing the oxLDL-induced formation of foam cell and therefore can protect vascular endothelial cells from inflammatory lesions [74]. Also, omentin seems to be involved in appetite regulation [75]. There was a direct relationship between serum levels of omentin and high-density lipoprotein (HDL) levels, while serum omentin was negatively correlated with anthropometric measures including BMI and WC [73]. The exact role of this adipocytokine in insulin sensitivity and glucose metabolism is yet to be clarified. However, it has been shown that the positive effect of omentin on insulin sensitivity is mediated through induction of AKT phosphorylation and enhancement in insulin-mediated glucose uptake [74, 76]. Recent studies reported that omentin levels are low in plasma and AT from patients with obesity and chronic inflammatory diseases. This indicates the protective role of omentin in obesity-related complications [74]. Moreover, the addition of insulin and glucose to human AT would reduce the expression of omentin in a dose-dependent manner [57, 73].

Vaspin

Vaspin (visceral adipose tissue-derived serpin, serpinA12) is a 47KDa protein belonging to the serine protease inhibitor family. It has been found in the vAT of the genetically obese Otsuka Long-Evans Tokushima Fatty (OLETF) rats [63, 71]. In human, vaspin is expressed in the AT, stomach, liver, SKM, skin, and pancreas [57]. Administration of vaspin to obese mice increased insulin sensitivity and glucose tolerance as well as reduced food intake [57, 63]. Further, vaspin administration can induce the expression of leptin, resistin, and TNF-α in scAT. The effect of vaspin on insulin and its receptor has not been well-understood. However, recent studies of HepG2 cells described that vaspin can bind to glucose-regulated protein (GRP78) and thereby make a contribution to insulin signaling [63]. Vaspin is mostly produced by the liver. Also, Klöting et al. found the expression of vaspin in 23% of vAT samples and in 15% of scAT samples [77]. Studies have found that vaspin levels positively correlate with anthropometric indices such as BMI, WC, and body fat percentage. Studies suggest a role for vaspin in the regulation of appetite. Different factors including gender, age, physical activity, and hormonal metabolism may affect vaspin levels.

WISP1

Wingless-type (Wnt) inducible signaling pathway protein-1 (WISP1, or Cyr61/CTGF/NOV) is an extracellular matrix-associated protein which includes matricellular proteins operating between cells and extracellular matrix. Acting as a pro-inflammatory adipokine, it is involved in biological functions and pathological processes. WISP1 levels are associated with high levels of IL-8 and with low levels of adiponectin [78, 79]. Fully differentiated adipocytes express WISP1 in visceral and subcutaneous AT which stimulates cytokine production in AT macrophage cells [79]. Furthermore, there is a direct relationship between serum and vAT concentrations of WISP1. Increased levels of WISP1 by inducing the expression of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IL-10 may be responsible for AT dysfunction and related metabolic disturbances [79].

Skeletal Muscle Related to Immunity and Obesity

Skeletal muscle (SKM) is an important organ which is able to secrete many proteins, low molecular weight molecules, and cytokines within the muscles (autocrine/paracrine) or to distant target organs (endocrine) [80]. Similar to AT, myocytes produce cytokines such as IL-6, IL-8, and IL-15 and other components such as FGF21, irisin, myonectin, myostatin, and myokines. By the secretion of myokines, SKM can

interact with the surrounding fat, bones, skin, and principal organs such as the cardiovascular system, brain, digestive tract, and glands [81].

Skeletal Muscle and Immunity

SKM is the largest tissue in the human body, which accounts for about 40% of total weight in non-obese population. The high metabolic activity of myocytes is essential for metabolism and energy homeostasis [80]. Emerging evidence indicates that similar to AT, inflammatory processes including immune cell infiltration occur in the early stages of obesity in SKM and may exert autocrine/paracrine effects on myocyte metabolic function [82]. Adipose depots within SKM are mainly found between myocytes and the surrounding muscle which are called intermyocellular/intermuscular AT (IMAT) or perimuscular AT (PMAT). Both IMAT and PMAT connect to myocytes and differ from scAT which would expand significantly in obesity and contract during weight loss [60]. Study of the SKM from obese humans revealed infiltration of immune cells such as macrophages and T cells. Accumulation of macrophages and T lymphocytes within adipose depots can form crown-like structures around dead or dying adipocytes. These cells tend to polarize into pro-inflammatory phenotype [60]. It has been said that most macrophages in SKM are $CD11c^+$ and display M1-like phenotype. Additionally, pro-inflammatory markers such as TNF-α, IL-1β, and IFN-γ are increased and $CD4^+$ and $CD8^+$ T cells are amplified in SKM with obesity [60].

Inter-organ (Adipose Tissue-Muscle) Cross Talk

The concept of inter-organ cross talk is one of the most interesting subjects in recent years, and it has been shown that AT contributes to a multilayered network of paracrine and endocrine pathways which show activation in a number of tissues including the liver, muscle, pancreas, brain, and vascular system [80]. There is a negative cross talk between AT and SKM [69, 83]. Myocytes and adipocytes produce and secrete different kinds of metabolically active myokines or adipokines, and adipomyokines, which are released from both SKM and AT, affecting the organ in a paracrine or autocrine manner. Furthermore, these proteins have endocrine effects which contribute to a bidirectional cross talk [69]. Gene expression of these components is changed in obesity, so this alteration can affect the circulating pool and the cross talk between AT and SKM [69, 84]. It has now been firmly established that adipocytes and myocytes in obese patients release pro-inflammatory factors, adipokines, and cytokines in a manner different from lean individuals [60]. Accumulation of lipids and their metabolites may negatively affect myocyte metabolic pathways [23]. It is well-known that lipid concentration is one of the strong indicators for the evaluation of insulin sensitivity in myocytes, adipocytes, and hepatocytes [23, 85]. A pro-inflammatory environment (DIO) can induce collagen synthesis and muscle fibrosis while inhibiting insulin signaling [86]. Moreover, IR can influence joint organ system by inhibiting the release of matrix metalloproteinase [23].

Adipocytokines

As we mentioned before, adipocytokines can affect myocytes. In the context of cross talk between AT and SKM, immune cells can through local autocrine/paracrine activity enhance SKM inflammation as seen in obesity and IR [80]. Below, we highlight the functional role of adipocytokines considering its relation to obesity.

Leptin

Leptin interferes insulin signaling in SKM [87]. AKT phosphorylation in human myotubes is increased with leptin [88]. Like scat, SKM produces leptin [60]. Emerging evidence indicates that leptin cooperates with the branched-chain amino acid leucine in the regulation of protein metabolism in SKM. Additionally, leptin-mediated activation of the mitogen-activated protein kinase-extracellular signal-regulated kinases (MAPK-ERK) pathways in some parts of hypothalamus plays a critical role in glucose uptake in red-type muscle and in whole-body glucose utilization [60].

Adiponectin

Adiponectin through different mechanisms helps in the regulation of lipid metabolism in SKM [60]. Studies of SKM show that high levels of adiponectin induce the expression of MKP-1, while its depletion leads to oxidative metabolism by upregulating MAPK-1 [89]. A low adiponectin state in HFD-fed mice led to a reduction mitochondrial biogenesis in SKM [90]. It is consistent with this finding that the expression of adiponectin negatively correlates with obesity through increasing free fatty acid levels [89]. On the other hand, adiponectin secreted by muscle cells acts in an autocrine/paracrine manner which is increased by mechanical and metabolic stressors as well as following lipopolysaccharide (LPS) injection [91].

Resistin

Resistin driven by AT is proposed to link between obesity to IR [92]. In SKM, resistin can decrease fatty acid uptake and metabolism through several mechanisms. First, it can reduce cell surface fatty acid translocase (FAT/CD36) content, the expression of the fatty acid transport protein (FATP1), and phosphorylation of AMPK, and acetyl-CoA carboxylase (ACC), without any change in cell viability. Second, resistin can reduce glucose uptake, oxidation, and synthesis of glycogen independent of AMPK by interfering with insulin receptor substrate-1, Akt1, and GLUT4 translocation. Third, it can reduce the expression of *suppressor of cytokine signaling 3* (SOCS3) [60, 92]. Moreover, it has been shown that resistin also reduces insulin signaling in myocytes via suppressing myogenesis and stimulating the proliferation of myoblasts in C1C12 myotubes [92].

Chemerin

Chemerin is a myoadipokine which is associated with obesity, T2DM, and metabolic syndrome. Overexpression of chemerin was associated with worsened IR in SKM of mice fed with a HFD. The effect appeared to be mediated by a reduction in insulin-mediated AKT phosphorylation [93]. It has been shown that chemerin can promote proliferation of muscle cells through ERK1/2 and the mechanistic target of rapamycin (mTOR) signaling pathways [60]. Additionally, the expression of chemerin is altered in WAT and SKM of the obese/diabetic mouse model. It indicates the influence of chemerin on glucose homeostasis and the metabolic derangement related to obesity and T2DM [60, 87].

Visfatin

Visfatin and leptin play a coordinated role in various functions. On the other hand, leptin can induce the production of visfatin by the regulation of MAPK and PI3K pathways [60]. It has found that visfatin is produced in higher levels by the cells in SKM than by those in AT especially in vAT. Therefore, visfatin can affect SKM growth and metabolism. On the other hand, visfatin can promote the transportation of glucose in skeletal muscle fibers via expression, translocation, and glucose uptake promotion [63]. One of the possible mechanisms for visfatin action is the induction of glucose uptake in SKM via Ca^{2+}-mediated phosphorylation of AMPKα2 [94].

Myokines

It is well-known that SKM can act as an endocrine organ which is able to express several myokines, among which some are secreted constantly but others in a temporally or context-controlled manner [95].

Myostatin

Myostatin is the first recognized myokine. It is a member of the TGF-β superfamily, preventing myoblast hyperplasia through the cell cycle progression and negative regulation of muscle mass [80, 95]. A recent study of mice showed that myostatin induces muscle hypertrophy as well as an elevated number of fibers by accelerating primary and secondary myogenesis and reduces total and intramuscular body fat. Higher levels of muscle mass can induce resting energy expenditure (REE) which acts against counter-regulatory consequence of leptin signaling [60]. Furthermore, myostatin is upregulated in obese SKM as well as in AT, and studies indicate that high levels of myostatin negatively affect growth in obese muscle by shifting the muscle ratio [60, 95]. Altered myostatin function may cause browning of WAT, increase in insulin sensitivity, and decrease in AT mass [60].

Interleukin-7 (IL-7)

Emerging evidence proposed IL-as a contributing factor to the link between SKM and lymphocytes of the immune system due to its essential role in survival and development of lymphocytes in the thymus [80]. It has been concluded that this myokine is found in mature SKM and may control the regulation of muscle cell development [80, 96].

Interleukin-8 (IL-8)

IL-8 is a low molecular protein that belongs to the cysteine-X-cysteine family of chemokines produced by macrophages and endothelial cells. In addition to acting as an angiogenic factor, IL-8 exerts significant chemotactic activity toward leukocytes [80]. Exercise induces the expression of IL-8 in SKM without any changes in plasma IL-8 levels. Muscle-derived IL-8 may have a local role. High levels of IL-8 produced by SKM especially in T2DM suggest that this myokine can be responsible for decreasing glucose disposal [60].

Interleukin-18 (IL-18)

IL-18 is expressed in SKM especially in inflammatory diseases. It engages different autocrine/paracrine mechanisms leading to the production of pro-inflammatory mediators importantly TNF-α [23]. IL-18 deficiency can lead to the accumulation of intramyocellular lipids (IMCL) due to its interference with AMPK pathway resulting in an impaired beta-oxidation in SKM [89]. Furthermore, it has been shown that IL-18 plays a crucial role in metabolism. IL-18 deficiency or IL-18 receptor deficiency in SKM has been associated with obesity, IR, and dyslipidemia [60, 61].

Fibroblast Growth Factor-21 (FGF21)

FGF21 belongs to the FGF superfamily which is involved in the modulation of cell proliferation, growth, differentiation, and metabolism. The secretion of this protein in the liver is increased in response to fasting, while BAT secretes FGF21 with noradrenergic stimulation [60]. It is well documented that FGF21has a predominant role in glucose uptake by SKM through increasing basal and insulin-stimulated glucose uptake in myotubes as well as enhancing GLUT1 mRNA abundance at the plasma membrane [95]. Another mechanism for enhancing glucose uptake is by potentiating insulin-stimulated glucose transport of isolated extensor digitorum longus muscle without any change in the phosphorylation of Akt or AMPK [42]. Interestingly, FGF21 has many functional similarities to adiponectin which regulates FGF21 function on energy metabolism and insulin sensitivity in SKM and the liver. Moreover, cold-induced FGF-21 in muscle could contribute to an activation of thermogenesis and increase in browning of WAT which may suggest that cross talk between SKM and AT can be mediated by this important myokine [95].

Apelin

Apelin is a myokine expressed in SKM. Exercise and obesity have been shown to increase the expression of apelin. Apelin enhances glucose uptake in C2C12 myotubes. Study of mice revealed the essential role of this myokine in skeletal muscle insulin sensitivity [97]. As expected, the administration of apelin to the mice improved insulin sensitivity. Reduced serum apelin levels negatively affected glucose uptake, Akt-phosphorylation, and fatty acid oxidation in SKM [69, 98].

Interleukin-6

IL-6 is one of the most important cytokines produced by different tissues, including SKM, AT, and the immune cells. It plays a crucial role in muscle performance. The adipokine IL-6 during contraction and chronic inflammation can induce IR in SKM [60, 95]. For example, IL-6 is secreted from skeletal muscle immediately after exercise and would accelerate fatty acid oxidation via the activation of the AMPK signaling pathway and glucose uptake [55, 60, 89]. IL-6 shows its biological effect through binding to the specific receptor and engaging signaling pathways. The role of IL-6 in body is confusing depending on the cellular microenvironment (muscle cell vs. immune cell). It can act as both a pro-inflammatory cytokine and an anti-inflammatory factor [60]. By being as an energy sensor, IL-6 plays regulatory roles in metabolism. In fasting state, increased release of IL-6 contributes to activation of AMPK and thereby enhances energy consumption especially glucose uptake in SKM, AT, and liver [57, 80]. Addition of IL-6 to SKM induced the expression of uncoupling protein 1 (UCP1), resulting in

increased lipolysis and fatty acid oxidation. Further, it has also been shown that this myokine enhances the uptake of basal glucose and translocation of the GLUT4 from intracellular compartments to the plasma membrane. The expression of IL-6 is more noticeable in type I skeletal muscle fibers. It has shown that IL-6 deficiency in SKM of mice can increase levels of free fatty acid transporters and intramuscular lipid content in type I but not in type II muscle fibers [60]. Studies suggest that IL-6 released from SKM can produce anti-inflammatory effects and enhance IR, especially under obesity-related metabolic problems [87]. It is suggested that there are two molecular mechanisms for the inhibitory action of IL-6 in insulin activity. One of them is the phosphorylation of the inhibitory Ser-307 residue of IRS-1, and the second one is the induction of suppressors of cytokine signaling 3 expression [95].

TNF-α

TNF-α is produced by adipocytes and SKM and may function in an autocrine manner to inhibit insulin signaling and glucose transport. TNF-α suppresses AMPK activity via TNF receptor 1. As a result, it would decrease fatty acid oxidation while increasing accumulation of intramuscular diacylglycerol and therefore causing IR in SKM [60]. High levels of TNF-α can cause IR by altering insulin signal transduction through enhancing serine phosphorylation of IRS-1. This protein mediates insulin signal transduction and pathways governing metabolic responses, such as GLUT4 translocation and glucose uptake in SKM. Documents which indicate the negative regulation of insulin action by TNF-α came from animal studies. Researchers showed that low levels of TNF-α improve insulin sensitivity in SKM and WAT through preventing obesity-related reductions in insulin signaling [23].

Conclusions

Under conditions of excessive caloric intake, AT undergoes different changes such as migration and activation of macrophages, natural killer cells, and lymphocytes. This will be followed by the overproduction of pro-inflammatory adipokines, such as TNF-α, IL-1β, IL-6, and other important adipokines, reduced capability of AT in free fatty acid storage, and the aberrant efflux of free fatty acids into the circulation. These molecular events lead to IR and obesity-related metabolic disturbances. Molecular links between obesity and associated metabolic disorders remain incompletely understood but may include chronic inflammation, particularly in AT and SKM. Inflammation may contribute to whole-body IR through the effects of adipokines on insulin sensitivity in different tissues, especially SKM. Myokines can affect myocytes, and in the context of cross talk between AT and SKM, the immune cells through autocrine/paracrine activity enhance SKM inflammation in obesity and IR.

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Nutrition, Immunity, and Neurological Diseases

19

Seema Patel

Contents

Key Points

- Inflammation generates reactive radicals, which cause acidosis.
- The acidification of body pH perturbs the neuro-endocrine-immune axis.
- Neuropathologies can occur from immune deficiency as well as autoimmunity.
- Diet can play a major role in homeostasis and its breakdown.
- Processed foods are acidogenic, so responsible for immune as well as neural pathologies.
- Diet with anti-inflammatory properties ought to be favored for immune and neural health.
- This chapter explores the link between diet, immune system, and neural system.

Introduction

The immune system, comprising lymphoid organs, lymphocytes, and cytokines, is vital for health and homeostasis [1]. Its balance can be tipped by pathogens, allergens, and mutagens. In fact, any form of stressors is capable of disrupting the immune system. The mechanism by which the immune system is provoked has been well-understood in the recent years. Despite their superficial differences, how stressors from diverse origins can affect the immune system in a hasty manner has been published [2]. Stressors of biological, chemical, or mechanical origin are perceived as a threat by the evolution-designed defense apparatus, following the recognition of PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns) in them. To contain the dangers posed by the stressors, inflammasomes, the multiunit inflammatory modules in the cytosol, are activated [3], enzymes behave aberrantly [4, 5], extracellular matrix (ECM)

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becomes acidic [6], and antimicrobial peptides are elaborated [7], so that the intruder can be trapped and neutralized. Activated inflammasomes elaborate pro-inflammatory cytokines/interleukins such as IL-1β and IL-18 [8]. Extracellular acidic pH activates proton-sensing proteins such as OGR1, GPR4, G2A, and TDAG8. The proton-sensing proteins affect actin polymerization/depolymerization [9]. Actin protein is indispensable for cell membrane integrity, chromatin structure, and gene expression [10, 11]. So, the above functions are hampered. Excess urease, a Ni^2+-containing hydrolase, can cause tissue injuries [12, 13]. Serine protease, cysteine protease, glycoside hydrolase, phospholipidase, and a gamut of other enzymes can wreak havoc in normal physiological functions. While this strategy is effective in dealing with some adversaries, the host body is battered as well. If the threats seldom arrive, body recuperates from this immune activation. But, in the face of recurrent onslaught of the stressors, the immune system is perpetually-activated, and it can cause chronic inflammatory diseases, including cancer. Food, pollen protein, plant alcohol, insect chitin, latex, cosmetics, leather, chemicals, drugs, and metals can be allergens [14, 15]. Allergenicity and autoimmunity results when the immune system turns against the host body itself, by elaborating oxidative cytotoxic T lymphocytes, cytokines, histamines, and antibodies [16, 17]. Due to the inextricable link of the immune system with the endocrine system, hormones, or the signaling molecules, behave erratically as a result of immune activation. Among other hormones, the master hormone estrogen shows dominance [5], causing tissue proliferation, among other pathological effects. In fact, in recent years, it has been recognized that excess estrogen is a hallmark of various forms of cancers.

A diverse range of neuropsychiatric ailments afflicts mankind. They basically result from abnormal, deficiency or excess, levels of neurotransmitters, such as serotonin, dopamine, acetylcholine, glutamate, epinephrine, adrenaline, and gamma-aminobutyric acid (GABA). These molecules transmit impulses through the synapses by synergy or antagonism. Neural homeostasis can be affected by the disruption of hypothalamic-pituitary-adrenal (HPA) axis as well as renin-angiotensin-aldosterone system (RAAS) [18, 19]. Angiotensin II, a vasoactive peptide, is the key effector of RAAS [20, 21]. Its role in the progression of Alzheimer's disease has been observed [22]. Estrogen prevents the adverse effects of RAAS [23]. Low estrogen level in postmenopausal females is the reason RAAS affects brain vasculature, inducing or worsening Alzheimer's disease [23]. The role of estrogen receptor alpha (ERα) as a regulator of renin has been confirmed [24]. Estrogen can be vital or lethal, based on the receptors it aligns to.

Therapies to Treat Immune Disturbance

Immunosuppressive drugs including non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are commonly used to suppress activated immune system in allergies and autoimmune diseases. They inhibit cyclooxygenase-2 (COX-2), thus reducing inflammation. The topical corticosteroids can cause immunosuppression and offer relief, but they can lead to side effects such as nausea, dizziness, itching, erythema, skin thinning and atrophy, and telangiectasia (dilated capillaries visible through the skin) [25–27]. Also, the immune suppression is a risk factor for infections with opportunistic pathogens such as *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Escherichia coli* [28].

Nutrition

Dietary habits vary among cultures and regions, mostly determined by the available ingredients. However, globalization of food habits is rising in keeping with the increasing industrialization, intensive agriculture, and transport. To cater to the consumer demand, chemical additives are being added to the food. The additives make food taste better and might improve their aesthetic

value and shelf life, but they can adversely affect the immune system. Mutagens as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) are generated during high-temperature cooking. Ultra-processed food is thus known to be carcinogenic to humans.

Flour and fat are acidogenic, which on regular consumption lead to acidosis [6]. Flour-containing foods such as pasta and noodles have a high glycemic index. Dairy products provide calcium, fat, and vitamin D. But the milk products such as butter and cheese may be harmful when they are consumed regularly [29]. The western diet which is rich in protein and low in plant products produces acids as sulfates and phosphate, which can cause bone loss [30]. Also, these acidogenic foods may aggravate multiple sclerosis [31]. Tyrosine, the precursor of dopamine, is linked to neurological diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis [32, 33]. Tryptophan is the precursor of serotonin, which alleviates depression [34]. So, tryptophan-rich diet is expected to improve the depressed mood. But, excess protein in the diet may not be beneficial for people with renal problems.

Meat products have dubious components including hormones and antibiotics. Chemical preservatives such as sodium acetate, sodium nitrite, potassium sorbate, sulfite, benzoic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and monosodium glutamate (MSG) have been identified to cause neurotoxicity and tumorigenicity [35]. Sulfites can elicit allergic responses, including angioedema, by mast cell degranulation or activation of kinin formation. Nitrate and nitrite have carcinogenic effects related to different types of cancers including ovarian [36], colorectal [37], and pancreatic [37], and renal cancer [38]. They also act as oxidizing agents converting hemoglobin to methemoglobin, which is unable to bind to and carry oxygen. BHA and BHT have been linked to ADHD in children [39]. Moreover, there are synthetic dyes and preservatives that can affect the behavior of children [40]. Supporting this, the removal of additives from food products improved behavioral issues [41]. Artificial dyes such as tartrazine, sunset yellow FCF, brilliant blue FCF, and indigo carmine inhibit cytochrome P450 (CYP) enzymes and P-glycoprotein, an ABC transporter family efflux pump [42]. MSG is a flavor enhancer associated with sleep-disordered breathing (apnea). Heavy metals including lead (Pb), cadmium (Cd), arsenic (As), nickel (Ni), mercury (Hg), bismuth (Bi), and tin (Sn) in food articles have been detected [43]. The organic forms of these metals cause neurological disorders [44]. With the rising popularity and increased consumption of canned and frozen foods which include large amounts of additives, the incidence of allergies and inflammatory diseases is soaring. The nutrients in the food seem to be lost by the addition of a multitude of anti-nutrient additives during the preparation.

Natural components such as vasoactive amines (histamines) are found in animal-based food products and can trigger an allergy in atopic people [45]. Plant- or animal-derived polysaccharides such as carrageenan, xanthan, gum arabic, chitosan, or alginate which are often used as emulsifier in foods are demonstrated to have inflammatory effects. These polysaccharides as a substrate for glycoside hydrolases [46] are critical for cleavage of the glyco-moieties, leading to their activation. Synthetic compounds, based on the structure of natural aromatic oils, are added to food. These fragrance compounds can influence hormones and neurotransmitters [47]. Pesticides such as organophosphate, organochlorine, and carbamate are found in vegetables. They interfere with immune and hormonal signaling and thereby cause health hazards [48, 49]. Seafood is getting tainted with xenobiotics, such as mercury, which are linked to different neuropathies [50].

The urban population is at risk of using chemical additives, while people in rural or remote areas are at risk of exposure to dietary toxicities. Plants can have anti-nutritional compounds like lectins, trypsin inhibitors, phytic acid, nonstandard amino acids, raffinose family of oligosaccharides (RFOs) (stachyose, raffinose, and verbascose), tannins, porosity-causing saponins, cardiac glycosides, and cyanogenic glycosides. Lectins can cause cell necrosis [51] and influence blood coagulation [52]. Chamorro people of Guam commonly con-

sume cycad (*Cycas micronesica*) seeds because of which neuropathologies such as Alzheimer's disease, Parkinson's disease, and *amyotrophic lateral sclerosis* (ALS) are more prevalent in this population. Flour substitutes contain the neurotoxin beta-N-methylamino-L-alanine (BMAA) [53]. *Lathyrus sativus* (grass pea) has an amino acid β-N-oxalyl-L-α, β-diaminopropionic acid which can cause paralysis on regular usage [54, 55]. Cyanogenic glycosides (linamarin and lotaustralin) from cassava (*Manihot esculenta* Crantz) root block cytochrome C oxidase and cause cyanide poisoning [56]. Bamboo species (*Bambusa vulgaris*) have been linked to death due to cyanide poisoning [57]. Another bamboo species, *Phyllostachys edulis,* has α-amylase inhibitors (orientin, isoorientin, vitexin, and isovitexin). Saponin glycosides can cause membrane leakage and hemolysis [58, 59]. Alkaloids can cause neurotoxicity depending on the dosage [60]. Among other mycotoxins, *Fusarium* fumonisin can exert neurotoxicity. The ergot fungus *Claviceps* elaborates the ergot alkaloid which can bind to the serotonin, dopamine, and adrenaline receptors, thereby affecting the nervous system [61]. The consumption of poisonous mushrooms can lead to neurological symptoms such as headache, hallucinations, anticholinergic toxidrome, and brain death [62].

As consumer awareness regarding diet is rising, food sector is undergoing radical changes. Paleo food, the food habits of humans during the Paleolithic Era (45,000–40,000 to 10,000 BP), has recently gained renewed interest [63]. This ancient diet included lean meat, fish, crustaceans, eggs, fruits, vegetables, tubers, and nuts but was free of cereals, dairy products, refined fats, salt, and sugar [64]. Prebiotics are nondigestible food ingredients that stimulate the growth of bifidogenic and lactic acid bacteria in the gut [65]. Probiotics which now constitute a huge economy help to maintain the beneficial gut flora balance [66]. However, there are controversies regarding the safety of probiotics for individuals with compromised gut. Microbiota inhabiting different niches of the human body, especially within the gut, has been proposed to influence cognitive functions. Therefore, the concept microbiota-gut-brain axis has emerged [67]. The microbes help to promote human health by suppressing pathogens, detoxifying carcinogens, and metabolizing complex dietary ingredients [68]. Dysbiosis paves the way for the development of the gastrointestinal barrier dysfunction, food antigen sensitivity, and inflammatory disorders. The perturbation in the microbiota-gut-brain axis may cause depression, schizophrenia, autism, and other neuropsychiatric disorders [69]. As a result, the recovery of nutrients from food wastes has been of increasing interest. Dairy whey, a milk industry waste [70], and soybean whey, a by-product of tofu manufacturing, are normally discarded. However, they have innumerable health-promoting components in them. Cereal bran, an agro-waste, has been shown to possess antioxidant, anticancer, and antidiabetic properties. Bran components such as tocotrienol, γ-oryzanol, dietary fibers, and β-glucan promote human health [71, 72]. Polysaccharides and oligosaccharides from unconventional sources, including seaweeds [73], have been identified as therapeutic components with proven applications in tissue engineering, drug delivery, and wound healing. Oligosaccharides as prebiotics have got immense credence [74, 75]. Food industry and agricultural wastes are processed by fermentation into nutrient-rich foods [76]. Fresh water organisms, such as alga spirulina [77] and krill [78], are being favored by consumers. Regional food products such as red yeast rice (the fermentation product of *Monascus purpureus*) [79], green tea [80], and traditional crops, including quinoa (*Chenopodium quinoa*) [81], and chia (*Salvia hispanica*), are finding global popularity. Even plants previously deemed as weeds, for example, purslane (*Portulaca oleracea*) and dandelion (*Taraxacum officinale*) are being increasingly consumed. Also, there is a rising interest towards regional and exotic foods such as truffles [82, 83], *Hibiscus sabdariffa* [84], pumpkin seeds [85], rose hip [86], nopal (*Opuntia* sp.) [87], and huitlacoche (*Ustilago maydis* growing on corncob) [88] .

Phytochemicals such as phenolics, flavonoids [89], glucans, quinones, terpenes, alkaloids [90], lectins [91], and saponins are being used as complementary and alternative medicines (CAMs). Ginseng (*Panax* sp.) saponin Rg has been reported to be protective against the accumulation of amy-

loid β (Aβ) peptide, which is the main pathological process in Alzheimer's disease [92]. More interestingly, there are plants with dietary supplement usage such as *Echinacea* [93], *Silybum* [94], maidenhair tree (*Ginkgo biloba*) [95], *Moringa oleifera* [96], ginseng [97], *Ziziphus* [98], *Diospyros* [99], and *Pistacia* [100]. The immunomodulating effects of phytochemicals have been reviewed [101]. Ethnobotanical studies have frequently identified plant families including Zygophyllaceae, Ranunculaceae, Annonaceae, Apocynaceae, Piperaceae, Labiatae (Lamiaceae), Asclepiadaceae, Liliaceae, Ebenaceae, Magnoliaceae, Berberidaceae, Fabaceae, Rosaceae, Linaceae, Compositae, Actinidiaceae, Anacardiaceae, Araliaceae, Malvaceae, Myrtaceae, Poaceae, and Papaveraceae, as sources of medicinal phytochemicals [102]. Mushrooms as immunomodulators have been well-studied [103]. *Ganoderma lucidum*, *Inonotus obliquus*, *Lentinula edodes*, *Grifola frondosa*, *Trametes versicolor*, as well as species from *Pleurotus*, *Agaricus*, *Phellinus*, *Clitocybe*, *Flammulina*, *Antrodia*, *Cordyceps*, *Calvatia*, *Schizophyllum*, *Suillus*, *Inocybe*, *Lactarius*, *Russula,* and *Fomes* have been found to possess antioxidant, antidiabetic, cholesterol-lowering, anticancer, nephroprotective, and antimicrobial properties. *I. obliquus* (chaga) is regarded as a panacea-type mushroom [81]. Nootropic and anxiolytic potential of plants such as *Bacopa monniera*, *Albizzia lebbeck*, *Moringa oleifera*, *Argyreia speciosa*, *Pueraria tuberosa*, *Celastrus paniculatus,* and *Hypericum perforatum* have been reported. They influence cognitive function by modulating acetylcholine release.

Mediterranean food articles such as whole grains, fruits, nuts, vegetable, seeds, legumes, and herbs are regarded as alkalogenic, so are healthy [104–107]. However, it is not likely to be the ideal diet for everyone from different genetic background and lifestyle. Hence, the dietary pattern should be designed according to the individual's metabolic needs. A dietary component that is beneficial for someone might be offensive or even fatal for another. For example, while most people can eat nuts, ranging from groundnuts (peanuts) to tree nuts (cashew, walnut, pine nuts, etc.), some are allergic to them, and upon exposure, may develop itching, swelling, edema, asthma, or more serious effects [108, 109]. Lipid transfer protein (LTP) in the nuts has been identified to cause immune manipulation [108, 109]. Peanut lectins can trigger anaphylaxis by inducing blood coagulation. Fish and shellfish allergy can be explained by the pan-allergen tropomyosin [110, 111] as well as chitin, an immunogenic polysaccharide [15]. Interfering with the actin-myosin interaction is responsible for the allergic responses to these seafoods [11]. Wheat is a staple cereal for a majority worldwide. But an increasing number of people have gluten sensitivity. Gluten-rich grains lead to enteric inflammations such as celiac disease in the atopic population [112]. Gut-associated lymphoid tissue (GALT) regulates the elimination of pathogenic microbes and toxins while tolerating commensal organisms and food antigens. When this balance of restriction and permission is broken due to the aberration of the immune responses, a range of food allergies, autoimmune diseases, and infections occur [113]. The gluten-derived immunotoxic peptide can cause neurological problems, as well [114]. A key cause of food intolerances is enzyme deficiency, which is inborn or acquired. For example, people suffering from phenylketonuria (PKU), a genetic disorder, cannot metabolize phenylalanine. Similarly, genetic links of intolerances to other amino acids have emerged. Diabetes mellitus predisposes individuals to be insulin-resistant. Therefore, patients with diabetes mellitus are not recommended to consume glucose-rich food. Low intake of salt leads to a reduction in the proinflammatory cytokines IL-6 and IL-23 as well as an elevation in the anti-inflammatory cytokine IL-10 [115]. In a review [52], the authors present a detailed perspective on foods allergies.

Given the fact that neurotransmitters are amines synthesized from dietary amino acids such as tryptophan, tyrosine, histidine, and arginine, diet and neural health are closely-linked. Evidence shows that scanty tryptophan triggers depressive mood and pain, which appear to result from depleted serotonin [116]. Pellagra (a wasting disease) arising from tryptophan deficiency may be accompanied by dementia [117]. The

amino acid tyrosine is the precursor of dopamine, catecholamines, and norepinephrine, which activate the reward center of the brain [116]. In this manner, tyrosine-rich foods increase the resistance to stress. Regular intake of a high-fat diet alters brain neurotransmitter circuitry including dopamine signaling [118]. The ketogenic diet (high-fat, low-carbohydrate) is considered beneficial to metabolic disorders (obesity and diabetes) as well as to neural conditions such as traumatic brain injury, stroke, epilepsy, ALS, Alzheimer's disease, and Parkinson's disease [119]. Also, biotin deficiency can lead to neuropathologies such as seizures, mental retardation, and teratogenicity [120].

Discussion

Nutritional programming can be used as a strategy to deal with diseases. Vitamins (A, C, E and B6, and folic acid) and enzyme cofactors (iron, zinc, selenium, and copper) are the most commonly-used micronutrients as supplements. They have been shown to modulate immune responses through intricate pathways. However, in high doses they may cause toxicity. Vitamin D is a critical defender of the immune system that can be obtained by sun exposure [121]. A dietary supplement can improve the serum level of this vitamin, but its efficacy may be reduced especially in obese individuals.

Mutations in the profilin 1 gene *PFN1* are known to cause ALS, a fatal neuropathology [122–124]. These mutations interfere with the ubiquitin-proteasome and autophagy system, leading to insoluble aggregate formation, axon growth prevention, and cytoskeletal network modification. Also, altered protein levels of PFN1 result in Fragile X syndrome, a cause of autism [125].

Based on the evidence presented above, it can be claimed that nutrition can shape immune status. A less acidic, organic, minimally-processed food can prevent the activation of inflamma-

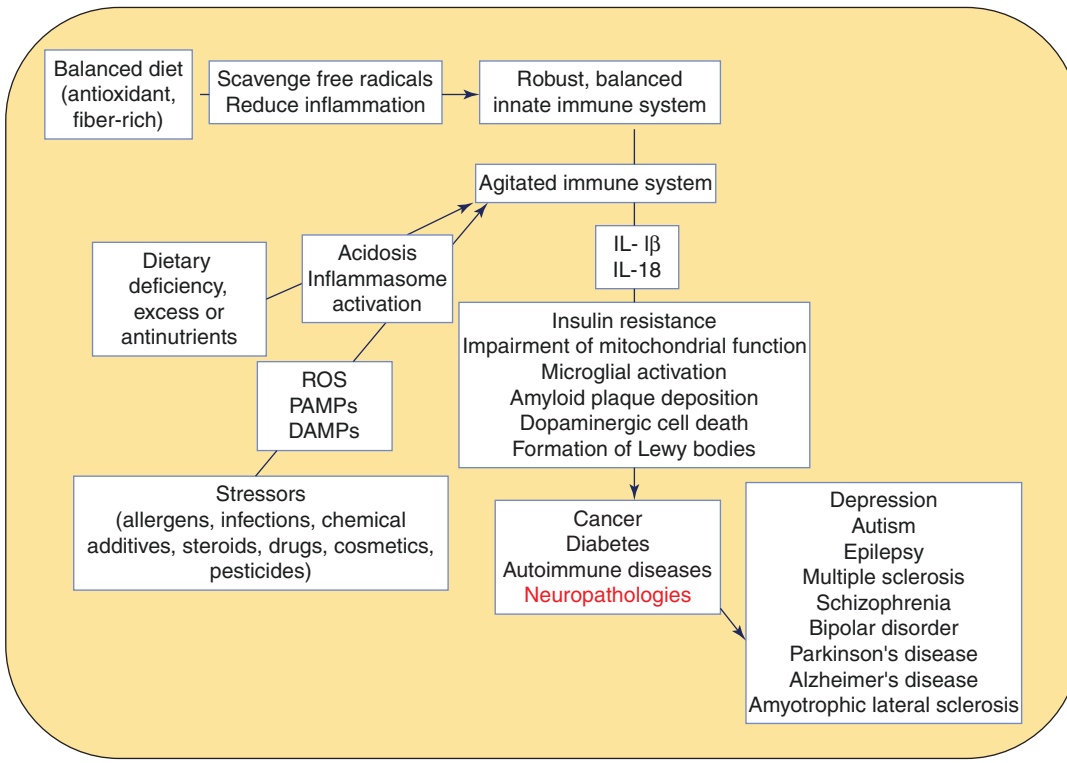

Fig. 19.1 The interaction between food, immune system, and neural health

somes, which can aid in maintaining the homeostasis of the innate immunity. A stable immune system is required to keep the body including the nervous system healthy.

The exposure to endocrine-disrupting chemicals by choice or accidentally can baffle the immune system. Cosmetics, drugs, vehicle exhausts, and pesticides are frequently encountered in the day-to-day life. Water and air pollution are commonly found worldwide. Nevertheless, public awareness about the hazards of these pollutants is not as much as it should be. Even if the healthiest foods are consumed, the immune system cannot behave normally when facing these stressors. If inflammasomes are activated, and the immune system becomes hyperactive, even the nutrients are suspected as enemies, due to the evolutionary-conserved pathogenic protein domains in them. For instance, dairy and meat-based foods can cause urticaria, dermatitis, and asthma in patients with cockroach allergy. It might be explained by the inability of a vigilant immune system to differentiate the sequence of amino acids in the allergen protein and the dietary protein. As a result, the dietary protein will be suspected as a threat, leading to cross-reactivity [126]. The evolution-designed strategy to tackle multiple homologous foreign antigens by few T lymphocytes is the allergic boomerang [127].

Nutrigenomics is a branch of genomics emerged to integrate nutrition information into molecular data, for the prevention and treatment of diseases by diet optimization [128]. Of note, nutrients can affect the gene expression. Accordingly, the personalized nutrition can be planned for an individual with a specific genotype. It is expected that nutrigenomics might one day assist in reducing the progression of neurological diseases by the restoration of immune homeostasis. Till that happens, a wholesome, low-processed diet and moderate lifestyle, free of pollutants, can prevent immune disturbances and hence neuropathies.

Figure 19.1 presents a schematic illustration of the role that a healthy diet might have in the maintenance of immune homeostasis. In contrast, stressors cause immune activation, leading to neuropathologies.

Conclusions

Healthy nutrition can help to maintain the immune system homeostasis. However, there is a broad range of stressors that provoke unwanted immune responses. Undernutrition and overnutrition can act as stressors. Also, host factors differ widely, so an ideal diet for an individual may not be the right choice for another. Also, an individual diet would require changes depending on the age, health status, and co-morbidities. In such a dynamic scenario, a balanced diet can help to restore the immune and neural health.

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Nutrition for Chronic Critical Illness and Persistent Inflammatory, Immunosuppressed, Catabolic Syndrome

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Contents

Key Points

- The hallmark of Chronic Critical Illness (CCI) and Persistent Inflammatory, Immunosuppressed, Catabolic Syndrome (PICS) is the continued breakdown of lean muscle leading to profound weakness, decreased capacity for rehabilitation, immunosuppression, and a propensity to develop recurrent nosocomial infections.
- With an aging population and continued advancements in critical care support systems, the prevalence of CCI and PICS is anticipated to increase.
- Unfortunately, therapies to mitigate or reverse CCI and PICS are limited.

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- A lack of randomized controlled trials has limited strong recommendations for nutrition support in the CCI patients.
- Nutritional therapies for PICS may be of biologic value.

Introduction

Over the past several decades, advances in intensive care unit (ICU) technology, support systems, care bundles, and evidence-based pathways have improved ICU survival [1]. Unfortunately, the cost of ICU survival is profound and leads to ventilator dependency, muscle weakness, immunosuppression, malnutrition, and a propensity for recurrent infections. Girard and Raffin in 1985 described chronic critical illness (CCI) as the need for an acutely ill patient requiring ongoing, and eventually, chronic support in the ICU setting [2]. The clinical definition of CCI is not well-established and quite variable, but it is an increasingly common phenotype in the elderly patients (>65) with multiple comorbidities who developed critical illness and sustained prolonged organ dysfunction [3–8]. Moore and colleagues have codified their definition of CCI to be greater than 14 days in the ICU with ongoing organ dysfunction as defined by SOFA, a C-reactive protein (CRP) concentration >150 Hg/dL and retinol-binding protein concentrations <1 mg/dL, immunosuppression crudely defined by a total lymphocyte count <0.80 × 109/L, a catabolic state defined by serum albumin concentrations <3.0 g/dL, creatinine height index <80%, and weight loss >10% or body mass index <18 during the current hospitalization. These criteria define persistent inflammatory, immunosuppressed, catabolic syndrome (PICS) which is now a common occurrence in survivors of multiple organ failure (MOF) in surgical ICU [8, 9].

Kahn and Iwashyna et al. projected that 5–7.6% of patients in the ICU develop CCI, accounting for greater than 380,000 cases, 107,000 in-hospital deaths, and over $26 billion in health-care costs [10, 11]. Additionally, it is this patient population that utilizes over 30% of ICU resources despite accounting for only 5% of ICU admissions [12]. Unfortunately, those who develop CCI that evolves into PICS do not get discharged home and long-term outcomes (mortality and morbidity) compared to those critically ill patients that are able to recover are dismal [10, 13–16]. Moore and colleagues described the PICS phenotype as a dramatic loss of lean muscle mass (catabolism) despite optimal nutrition associated with profound weakness, recurrent nosocomial infections (immunosuppression), and consequently development of decubitus ulcers, poor wound healing, sepsis recidivism, and poor long-term outcomes [1, 9, 17, 18]. PICS patients tend to be or become frail and suffer from significant pain, dyspnea, psychological distress, thirst, fatigue, delirium, and distress related to their protracted hospital stay [19–24]. CCI and PICS patients who survive have a poor long-term quality of life, with many suffering from depression, cognitive impairments, complex physiologic abnormalities, and chronic organ dysfunction, including neuroendocrine and immune dysfunction [3, 4, 23, 25–30].

Nutrition in PICS

The predominant phenotypic expression of PICS is cachexia that is surprisingly similar to that seen in patients with refractory cancer, major burns, and aging sarcopenia. Anabolic (and anti-catabolic nutrients), immune-modulating agents, pro-resolving mediators, protein and amino acid augmentation, and various feeding strategies that have shown promise in these populations may play a crucial role in rehabilitating PICS patients by mitigating each of the PICS categories: immunosuppression, inflammation, and catabolism.

Anabolic Supplements

Anabolic and anti-catabolism agents may be beneficial in mitigating the catabolism pillar of PICS. Herndon described numerous methods to mitigate catabolism in the pediatric burn population that may be of value in the PICS population, including (a) growth hormone [31], (b) intensive insulin therapy [32, 33], (c) oxandrolone [34, 35], (d) propranolol [36], and (e)

exercise programs [37]. These therapies have a net ability to be "potent anabolic agent and salutary modulators of post-traumatic metabolic responses" [31]. They would increase lean muscle mass and strength and bone mineralization and attenuate the hypermetabolic response to burn injury leading to a quicker recovery [32, 33, 38].

Interestingly certain branched-chain amino acid [BCAAs: LEU, isoleucine, and valine] supplementation results in a decreased muscle protein catabolism and increased protein synthesis [18]. Frank Cerra championed an idea of "septic autocannibalism" that occurred in MOF patients despite standard parenteral nutrition (PN) and recommended using BCAA to combat the ongoing catabolism [39]. In a PRT, Cerra demonstrated that the use of BCAA-fortified PN in surgical patients improved visceral protein markers of nutrition and nitrogen balance as well as absolute lymphocyte count that is one of the laboratory parameters of PICS. This study, however, failed to prove any mortality benefit, despite having a signal of providing strong nutritional support. As these BCAA formulas were expensive to make interest, they slowly died until recently where studies have linked leucine supplementation to target rapamycin complex1 (mTORC1) pathway [40, 41]. mTOR is a potent inducer of protein synthesis and can potentiate an anabolic effect when leucine binds to its receptor [42]. mTOR, unfortunately, is downregulated in the initial septic state making leucine supplementation ineffective [43]. Interestingly, what is not known is whether this persists into the CCI phase of PICS and whether the literature that supports leucine supplementation in aging sarcopenia and cancer cachexia could apply to PICS?

Immune-Enhancing Nutrition

Arginine is a semi-essential amino acid that is important for T-cell function and wound healing [44, 45]. Endogenous arginine production is insufficient during periods of metabolic stress (such as sepsis) and requires exogenous supplementation to restore maximal function of the immune system [46]. Furthermore, arginine is the chief precursor of nitric oxide and has been shown to increase protein synthesis and improve wound healing [47]. PICS patients because of their low-grade inflammation and immunosuppression typically cannot mount an effective inflammatory response to infection, and consequently, the unfounded controversy concerning arginine aggravating an acutely septic state is irrelevant. In fact, CCI-PICS patients have a persistent expansion of myeloid-derived suppressor cells that overexpress arginase 1 which depletes arginine. Arginine contributes to T-cell development by serving as a structural component of the T-cell receptor zeta chain [45, 48]. In an arginine-depleted state, T cells are impaired, both in quantity and quality, increasing immune dysfunction and the risk for nosocomial infections.

Pro-resolving Mediators

Specialized lipid mediators called pro-resolving mediators (SPMs) are a relative category of immune-modulating agents that have shown promise in treating and preventing CCI. Of the SPMs, resolvins reveal the most potential for resolving of inflammation. SPMs are derived from purified fish oil. They promote resolution of the dysfunctional inflammatory response. Serhan and colleagues have shown that SPMs decrease inflammation by halting leukocyte infiltration and activation and enhanced macrophage clearance of debris, bacteria, and apoptotic cells [49, 50]. SPMs, therefore, may potentially attenuate the dysfunctional immune and inflammatory response observed in CCI-PICS patients, which may promote earlier recovery to homeostasis in these critically ill patients. Further research is needed to determine the impact of SPMs on clinical outcomes before recommendations can be made for widespread use.

Benefits of Enteral Versus Parenteral Nutrition

Prospective randomized controlled trials (PRCTs) in the 1980s and 1990s comparing enteral nutrition (EN) to PN had a significant impact on clini-

cal practice. These trials demonstrated that patients receiving early EN had significantly fewer infectious complications, which would hopefully help the CCI/PICS population by decreasing the second hit phenomenon of nosocomial infections. A meta-analysis of eight PRCTs (six published, two not published) [51] provided a foundation that EN is preferred over PN in critically ill patients. In a recent meta-analysis, Marik and colleagues evaluated the effect of early (within 48 hours of critical illness) versus delayed EN in acutely ill (medical and surgical) patients and demonstrated a decrease in infectious complications in those patients who received early EN [52]. Hence, an early EN is preferred over delayed or no EN to decrease septic morbidity.

The Role of Protein Supplementation

Sarcopenia is progressive loss of muscle mass and has been commonly described in the aging population. In fact, sarcopenia can also be a consequence of CCI and PICS, due to acute and chronic inflammation and associated proteolysis, malnutrition, and immobility with muscle disuse [53]. During periods of physiologic stress, the body mobilizes and then catabolizes large amounts of protein from muscle [54]. In sepsis and blunt trauma, resting energy expenditure peaks at around 5 days, but can continue for up to 12 days, and patients may lose up to 16% of total body protein [55–57]. The hypermetabolic response to burn is associated with increased energy expenditure, insulin resistance, immunodeficiency, and whole body catabolism that may persist for months after the initial injury [31].

Paddon-Jones and colleagues established that a daily protein consumption of 0.8–1.5 g/kg/d potentially slows or prevents muscle protein catabolism in patients with sarcopenia [58–60]. Morely and colleagues and the Society of Sarcopenia, Cachexia, and Wasting Disease recommended >1.5 g/kg/d of protein in combination with exercise and supplemental amino acid leucine [61]. Leucine is an amino acid that can stimulate the mTOR pathway to increase protein synthesis (anabolic) and inhibits protein breakdown (anti-catabolic). Moreover, Wolfe and Deutz 2013 described an "anabolic response" where higher protein supplementation suppresses endogenous protein breakdown. The anabolic response is a measure of fractional synthetic rate (FSR) minus the protein breakdown and was higher with a greater protein delivery [62]. PICS patients, by definition, are in a chronic catabolic state with loss of lean body mass and optimizing protein delivery in this population may increase the FSR.

Over the past decade, observational studies have shown a clear benefit of optimizing the delivery of protein over nonprotein calories [63, 64]. Weijs and colleagues showed that greater protein delivery had a survival benefit over nonprotein calories and that energy overfeeding in the form of lipids and carbohydrates was associated with increased mortality [40, 65]. Allingstrup and colleagues, in another observational study, reported that greater protein provision (more than 1.46 g/kg/d) was associated with lower mortality, as compared to 1.06 or 0.79 g/kg/d [66]. More recently, Compher and colleagues demonstrated that increased protein delivery was associated with a significant survival benefit in nutritionally high-risk patients, as determined by the Nutrition Risk in the Critically Ill (NUTRIC) score of greater than 5. Compher concluded that greater nutritional and protein delivery is associated with lower mortality and faster time to discharge alive in high-risk and longer stay in ICU patients [63]. CCI and PICS patients are generally considered to be at nutritional risk.

How much protein is required to offset proteolysis and restore muscle synthesis during and after critical illness? Historically, more than 1.2 g/kg/d protein was recommended for critically ill patients [18, 65, 67]. In the general critical care patient, current guidelines recommend at least 1.5 g/kg/d protein supplementation, but this value may be different depending on the critical care phenotype. For example, in burn patients, Herndon and colleagues recommended up to 2 g/kg/d protein to compensate for the ongoing catabolic insult after burn injury [68]. The optimal protein dose in a CCI and/or PICS patients is

unclear, but pathophysiologic rationale and inferences from observational trials suggest at least 1.5 g/kg/d, if not more, protein can be provided to this patient population. Studies to elucidate optimal protein type, dose, and delivery method are needed before stronger recommendations can be made for the PICS population.

Conclusion

CCI and PICS are the cost of ICU survival and the underlying pathophysiologic mechanisms contributing to their phenotype are being unraveled. Unfortunately, CCI and PICS lead to significant morbidities, many of which are difficult to reverse. Identifying which patients are at risk of CCI and PICS is of utmost importance. Early commencement of therapeutic strategies may mitigate progression to CCI and PICS. When PICS is established, combating the pillars will require multiple modalities and a multi-professional team. Nutrition is merely one modality and outcomes to determine the impact of nutritional interventions remain to be determined. While inferences from observational trials provide some insight into the management of PICS, the value of anabolic agents, immune-modulating therapies, including SPMs, early EN, and high-protein dose, to combat PICS remains to be fully determined and explanatory trials are sorely needed.

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Nutrition, Immunity, and Autoimmune Diseases

21

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Key Points

- Autoimmunity is a multifactorial condition which is caused by autoreactive B cells and T cells that produce high levels of cytokines and pro-inflammatory elements after cross-reactivity with autoantigens, defect in tolerance, and lymphoproliferative dysregulation.
- Autoimmune diseases are divided into two subgroups based on the affected tissues/organs including tissue-specific and systemic autoimmune disorders.
- B cells and T cells both take part in tissue-specific autoimmune diseases such as autoimmune thyroiditis, type1 diabetes, multiple sclerosis, inflammatory bowel disease, and autoimmune liver diseases.
- Production of autoreactive antibodies against various autoantigens is a prominent cause of systemic autoimmune disorders including systemic lupus erythematosus, Sjogren's syndrome, and rheumatoid arthritis.
- Nutrition and diet play an important role in developing an autoimmune condition so that a high calorie diet (rich in saturated fats or processed food and low in fibers) may disturb the immune system to properly discriminate self from nonself and lead toward autoimmunity.
- An anti-inflammatory diet which contains nutrients limited in pro-inflammatory compounds (vitamin D, antioxidant, and minerals such as zinc) can effectively attenuate the risk of autoimmunity through decreasing pro-inflammatory cytokines (IFN-γ) and increasing regulatory T-cell activities.

Introduction

In normal conditions, the immune system is able to recognize and combat potentially harmful foreign agents invading the body. When the immune system is in abnormal conditions, the body may lose the ability to distinguish host from nonhost. This results in the initiation of immune responses that may present a potential threat to the host. At the beginning of the twentieth century, Paul Ehrlich described an overactive immunity against host as "horror autotoxicus." Then, the concept of auto-reactivity was strengthened by the observation of autoantibodies in 1957 [1].

Autoimmunity refers to conditions where the immune system reacts against autochthonous antigens. Varieties of unwanted immune reactions can evolve by either a foreign or a native antigen. Adaptive immune responses involving both B cells (autoreactive antibodies) and T cells (autoreactive T cells) play a paramount role in the pathophysiology of autoimmunity [2]. Other factors that may contribute to autoimmunity include the innate immune responses, genetic and epigenetic factors, microbial allergens, and environmental factors (smoking or UV light). In fact, the machineries of autoimmunity involve a myriad of cellular and molecular pathways. As a result, it is not easy to understand which the main etiology for autoimmunity is. Of particular interest to the present chapter is that autoimmunity is characterized by a variety of immune abnormalities such as aberrant control of regulatory T cells, defective self-tolerance, impaired immune cell development, and dysregulated immune signaling pathways [3]. As a matter of fact, autoimmunity does not happen all at once. But it is a multifactorial event that takes years to be diagnosed when circulating autoantibodies appear in the peripheral blood [4].

Overall, autoimmunity is more common in women than men [5]. Women constitute about 78.8% of autoimmune cases. This would open the possibility that sex hormones including estrogen and progesterone play role in autoimmunity as well [6].

Autoimmune reactions have been observed in more than 80 disorders [7]. Among which, only 15 disorders appear to directly result from immune reactions [8].

Autoimmunity is commonly accompanied by inflammation as reflected in excess production of cytokines and chemokines. Inflammatory processes keep the body vigilant against nonhost. On the other hand, they can contribute to the aggravation of autoimmune diseases [9].

As the incidence of autoimmune diseases has increased, so has interest in investigating the eti-

ology of these diseases. Research has linked autoimmunity to different environmental factors including pathogens, vaccination, and chemical, solvent, and detergent substances. It is thought that with increased use of vaccines, detergents, and antiseptics, the immune system is less likely to be exposed to foreign antigens as before. This would increase the sensitivity of the immune system against harmless elements and promote autoimmune reactions [10, 11]. Furthermore, nutrition and habitual diet are also known as important factors that can affect the incidence of autoimmunity. Of note, a diet rich in saturated fats, cholesterol, sugar, salt, protein, and highly processed foods communally known as "Western diet" can lead to the initiation and/or exacerbation of inflammation and autoimmune diseases [12].

In summary, autoimmunity would be influenced by hereditary as well as environmental factors. A healthy diet can assist the immune system to achieve more control over discrimination of self from nonself.

Mechanisms of Autoimmunity

Autoimmune reactions happen because of a failure of self-tolerance. The major responsibility of lymphocytes is immune recognition. For that purpose, lymphocytes express specific receptors to distinguish self from nonself. There are broad repertoires of lymphocyte receptors resulting from recombination of surface proteins. High levels of diversity are particularly evident for recombination-activating enzymes called RAG-1 and RAG-2 and hypermutation in B cells [13]. Encountering self-antigens during development and maturation would help lymphocytes to become familiar with self-materials and discriminate them from foreign agents. Genetic background defines the type of immune responses in the face of stressors that can be protective or deleterious. Autoantibody production can be directly related to HLA haplotypes which are shared between members of a family. An HLA haplotype can effectively predict the risk of autoimmunity. Generally, there is association between different autoimmune disorders so that the probable incidence of one autoimmune condition allows the risk of other autoimmune disorders to be increased. For example, one of the genes in lymphocytes is PTPN22 (protein tyrosine phosphatase gene). PTPN22 is highly associated with rheumatoid arthritis (RA), type 1 diabetes (T1D), autoimmune thyroiditis, and systemic lupus erythematosus (SLE) [14, 15]. In addition, regulatory genes such as the genes encoding CTLA-4 are also known to affect the susceptibility to autoimmune diseases [16]. Autoreactive T cells recognize self-antigens through CD4 molecules. Therefore, it has been proposed that MHC-II molecules play a prominent role in making an autoimmune reaction by presenting native peptides to CD4+ T cells [17]. Additionally, environmental factors including drugs and infections take part in autoimmunity. α-methyldopa and hydralazine can induce hemolytic anemia and lupus-like disease, respectively [18, 19]. The major mechanism in inducing autoimmunity following infection is the occurrence of molecular mimicry between environmental antigens and self-antigens which are recognized by lymphocytes. Other mechanisms include viral persistence, epitope spreading, dysregulation of immune hemostasis, and autoinflammation [20, 21]. Cross-reaction between host immune components and invading pathogens results in the initiation of innate and adaptive immune responses accompanying inflammation. Molecular mimicry promotes the production and survival of autoreactive B and T cells. Overproduction of costimulatory factors is another autoimmune mechanism that disturbs self-tolerance [22]. The other mechanism is about superantigens that are able to activate T cells and make them to react against the host [23].

It has not yet clear that lack or excess of a nutrient can be the initiator of an autoimmune condition. However, nutrition and dietary factors have been proven to participate in autoimmunity, as being protective or inducer. In each condition, there is a triggering antigen that can activate B cells to produce complement-activating immunoglobulins and stimulate NK cells to enhance phagocytosis. Autoreactive antibodies seek to destroy host cells like red blood cells (RBCs). They can also react against surface receptors such as thyrotropin receptors and thereby disturbing thyroid stimulation. Additionally, T cells (cytotoxic and helper) have been related to auto-

immunity. For example, in T1D, Th_1 cells are mostly responsible for organ damage and autoimmune diseases [24]. Pro-inflammatory cytokines including TNF-α, IFN-γ, IL-6, and IL-17 are generally produced during autoimmune responses and play a prominent role in pathophysiology of this condition. A brief scheme of the tissue-specific and systemic mechanisms of autoimmunity is presented in Fig. 21.1.

Autoimmune Diseases

Autoimmune responses occur against a specific antigen in a particular tissue. For instance, when the immune system assaults insulin-producing cells, β-cells, T1D will develop, or when the immune system attacks myelin sheath surrounding the nerve fibers, multiple sclerosis will happen. These kinds of autoimmune reactions are usually specified to the category of organ-specific autoimmune diseases which are also known as tissue-specific autoimmune disorders. Conversely, the immune response against self-antigens may be systemic and involves many different cells and tissues (e.g., systemic lupus erythematosus, in which the autoimmune reactions affect various tissues and organs containing skin, joint, kidneys, heart, and brain) [25, 26]. Therefore, when multiple organs and tissues are distinguished as suitable targets for the immune cells and afflicted by immune attack, the systemic autoimmune disease occurs.

Systemic Autoimmune Diseases

Systemic autoimmune diseases (SADs) are a group of connective tissue diseases characterized by the presence of reactive autoantibodies against a variety of autoantigens including nuclear components, cell surface molecules, and intracellular matrix proteins. Table 21.1 illustrates SADs, and related etiology and complications.

Although the exact cause of SADs remains unclear, several immunological mechanisms have been implicated along with genetics, infections, and environmental factors. SADs are sometimes difficult to be distinguished due to overlapping signs and symptoms. Food can be a source of chemical reactant which has been implicated in the acquisition of SADs. There are some examples where the presence of a particular substance in diet might possibly be related to a systemic autoimmunity. Sometimes components of the diet may influence the development of the disease, and sometimes abnormal immune responses may also be due to a deficiency of a specific substance.

SADs are one of the leading causes of death and disability. With the use of chemical drugs, immunotherapies, and other therapeutic strategies, the outcome of this group of diseases has greatly improved, but there is still no definite cure for SADs. However, knowledge of the pathogenesis is important for a better understanding of SADs, and their diagnosis and treatment.

Organ-Specific Autoimmune Diseases

It is well established that organ-specific autoimmune diseases have genetic predisposition and occur in families. It has been known that HLA-DR3-DQB1∗0201 is a haplotype of MHC that has considerable effect on the occurrence of various organ-specific autoimmune condition [27]. In addition, environmental factors such as infections and diet are also associated with autoimmune diseases. Both arms of the adaptive immune system including T cells and B cells recognize self-antigens presented on the surface of organ cells and destroy them. Autoantibodies against organ-specific antigens have been also detected in patients with organ-specific autoimmune disorders including autoimmune thyroiditis, T1D, multiple sclerosis, celiac disease, inflammatory bowel disease, and autoimmune hepatitis. For instance, the target tissue for immune reaction during autoimmune thyroiditis and T1D are thyroid and the islets of Langerhans, respectively. Breakdown of immune tolerance causes lymphocyte infiltration and autoantibody production which lead to inflammation and tissue damage. Immunosuppressive drugs and complementary diet have been found effective for treatment of autoimmunity [28]. Table 21.2 provides a summary of important organ-specific autoimmune diseases and related pathophysiology and complications.

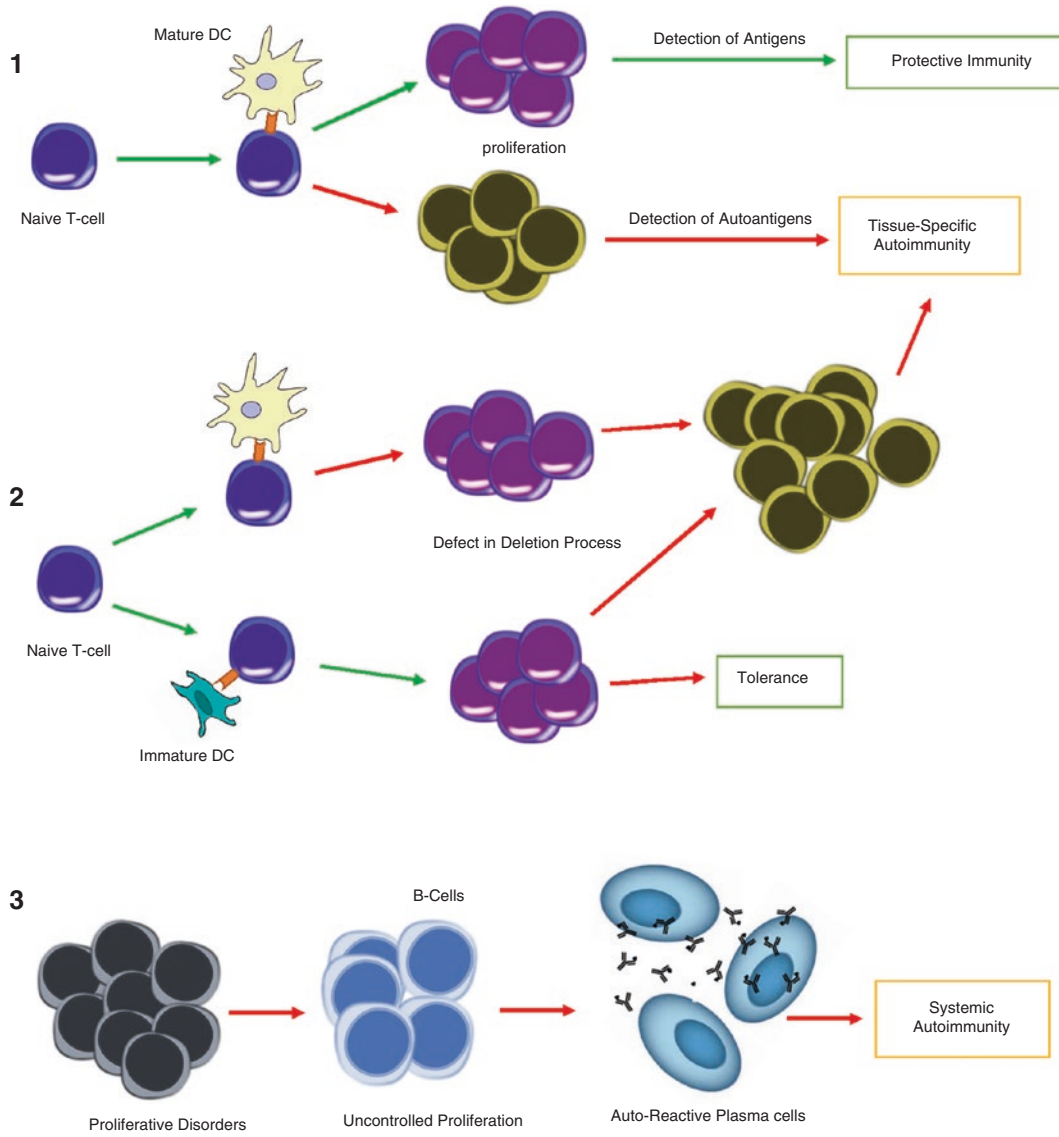


Fig. 21.1 Mechanisms of autoimmunity. Naive T cells are stimulated with foreign antigens presented by mature dendritic cells (DCs) leading to normal cell-mediated immune response. These immune responses may act against self-antigens in various tissues (cross-reactivity) and cause tissue-specific autoimmunity (1). During thymic deletion, autoreactive T cells are deleted. Defection of these processes may lead to develop autoreactive lymphocytes, which react against autoantigens in several tissues. In addition, stimulating naïve T cells by immature DCs may result in immune tolerance due to deletion or anergy mechanisms (2). Autoreactive B cells can be generated by several mechanisms such as lymphoproliferative disorders and imperfect mechanism of deletion. These cells differentiate into autoantibody-producing plasma cells. Autoantibodies circulate through blood, react against autoantigens, and result in autoimmune systemic diseases (3)

Effect of Nutrition on Autoimmune Diseases

Several studies have identified the role of diet in initiation and progression of as well as in protection against autoimmunity. However, the relationship between nutrition and autoimmune diseases remains elusive. There is no specific diet for the treatment of autoimmune diseases.

Table 21.1 Systemic autoimmune diseases: etiology and complications

Diseases names	Predisposing factors	Pathophysiology	Complications
Systemic lupus erythematosus (SLE)	HLA DR2/DR3, complement deficiencies, infections, sex, age, and race	DNA breakage by UV, cell apoptosis by caspase 1	Glomerulonephritis, cardiovascular disease, discoid lupus, psychiatric lupus, arthritis, miscarriage
Rheumatoid arthritis (RA)	HLA DR1/DR4, smoking, obesity, sex, and age	Citrullination of proteins in joint fluid	Arthritis, osteoporosis, changing shape of joints
Sjogren syndrome (SS)	HLA Dw3 and 138, a family history of autoimmune disease, number of pregnancies	Cell apoptosis by caspase 3	Eye and mouth dryness, arthritis, neuroendocrine disorders
Systemic (scleroderma) and limited (CREST syndrome) sclerosis	HLA DR5/DR1, sex, age, environmental factors	Increased collagen deposition, perivascular mononuclear cell infiltration, and vascular abnormalities	Raynaud's phenomenon, thickening and tightness of the skin and of subcutaneous tissue, Tendon friction rubs, nail fold capillaries
Mixed connective tissue diseases (MCTD)	HLA DR4, sex, age, number of pregnancies, environmental factors	Autoantibody directed against select components of the spliceosome, B lymphocyte activation, and CD4 and CD8 T lymphocyte participation	Overlap syndrome, miscarriage, arthritis, cardiovascular, and renal complications
Inflammatory myositis/ dermatomyositis	HLA DRB1, and -DQA1, metabolic disorders, infections	Autophagy, apoptosis, and innate and adaptive immune mechanisms lead to muscle weakness	Muscle weakness, skin lesions, arthritis
Antiphospholipid syndrome (APS)	HLA DRB1/DQB1 and DQA1, other autoimmune diseases, age, sex, and infections	Autoantibodies act against phospholipids and cause intravascular coagulation	Thrombosis, recurrent miscarriage, bleeding, lung embolism, cardiovascular involvement, neuropathies
ANCA-associated vasculitides	HLA DR1, DQw7 or DR8, smocking, chronic diseases	Inflammatory lesions of the upper and lower airways and pauci-immune glomerulonephritis with and without granuloma formation due to antineutrophil antibodies	Glomerulonephritis, cardiovascular disorders, neurological complications. aneurysms, vision loss, or blindness

Table 21.2 Tissue-specific autoimmune diseases: etiology and complications

Diseases names	Predisposing factors	Pathophysiology	Complications
Autoimmune thyroiditis	HLA-DR3 and -DR5, A4 promoter genes in CD8+ T cells, viral infections, sex and age	Autoantibodies against thyroid-specific proteins, CD 8+ T cytotoxicity	Inadequate thyroid hormones (T3 and T4), increased amount of TSH, blood pressure, inflammation, fatigue, constipation, muscle cramp, depression, dry skin, and weight gain /lose weight
Type 1 Diabetes	HLA-DR3 and -DR4, non-HLA genes including IDDM2 and IDDM12, infant diet, hygiene status, obesity, sex and age	Islet cell destruction, pancreatic β-cells deterioration, malfunction, and Fas-mediated apoptosis	Hyperglycemia
Multiple Sclerosis	HLA-DQ6 and -DR15, sex and age	Demyelination of CNS, blockage of signal transduction, T cell cytotoxicity, autoantibodies against MBP and MOG	Blindness, muscle malfunction, damaged sensation, and inefficient harmony in the body
Celiac Diseases	HLA-DQ2 and -DQ8, sex, age, environmental factors	T cell, B cell, NK cell against gliadin peptides, IL-15 production, antitransglutaminase antibodies	Malabsorption syndrome including diarrhea, emaciation, aphthous, and stomatitis
Inflammatory Bowel Disease (IBD)	HLA DR2, DR9, and DRB1∗0103, environment, and microbes	Autoantibody directed against epithelial cells in intestine and goblet cell glycoproteins Th17 lymphocyte activation	Abdominal pain, diarrhea, intestinal bleeding, severe internal cramps, vomiting, anemia
Autoimmune liver disease (ALD)	DRB1∗0301 and DRB1∗0401, infection, sex, age	T-cell mediated injury of the liver, IgG production against liver compartments, breakdown of the regulatory pathways of CD28-IL-2 and IL-12/STAT4, inflammatory cytokines (IL-17, TNFα, and IL-22)	Liver damage, fever, arthralgia, anorexia, fatigue, muscle aches, jaundice, and cirrhosis

* Class I and class II alleles are designated by the locus and gene name separated by a hyphen, followed an asterisk (HLA-DRB1*). The first two digits after the asterisk defines the allele of the gene and this number frequently but not always matches the serological type (HLA-DRB1*03); the next two digits define the subtype of the allele (HLA-DRB1*0301 or HLA-DRB1*0302) (http://www.anthonynolan.org.uk/HIG/lists/nomenlist.html).

High-Calorie Foods

High-calorie foods, especially processed foods, might cause weight gain and impair the proper functioning of the body and the immune system. In fact, fat accumulation in the body may increase the risk of some complications such as insulin resistance and hypertension. Therefore, the immune system deviates from the ideal protective state to the systemic and tissue-specific inflammation in some cases [29]. There are some kinds of inflammatory substances including C-reactive protein (CRP), leptin, resistin, and cytokines such as TNF-α and IL-6, which are produced by adipose tissue as an endocrine organ. These substances, also known as adipokines, promote inflammatory reactions. Therefore, fat accumulation in the body can influence the immune system in direct and indirect ways [30]. Leptin which routinely controls energy balance is able to induce T-cell proliferation and Th1 activation that are involved in the development of autoimmunity [31]. Therefore, an anti-inflammatory diet limited in pro-inflammatory compounds and rich in anti-inflammatory compounds can be efficient in controlling or even treating autoimmune diseases. Inflammatory signals originated from high caloric diets stimulate $CD4^+$ T cells and promote Th17 responses [32].

Animal studies suggest that energy restriction improves renal function in mice with SLE and increases longevity. In other words, caloric restriction can delay the onset of glomerulonephritis and kidney inflammation in animal studies [33]. There is no direct evidence confirming the same results in humans. However, disease activity in premenopausal women with lupus has been associated with body mass index (BMI) [34].

Moreover, obesity and metabolic syndrome can propel individuals toward inflammatory autoimmune diseases (Fig. 21.2). In particular, the association between obesity and autoimmune gastroenteropathies has been well-demonstrated so that alterations in adipose tissue are seen in patients with Crohn's disease. Furthermore, there is clear evidence that patients with early-diagnosed RA frequently report metabolic syndrome. In addition, energy restriction can reduce levels of circulating autoantibodies, pro-inflammatory cytokines, prostaglandin E2 (PGE2), and cold agglutinins in patients with autoimmune vasculitis [35].

Obesity is a culprit of T1D. Hyperinsulinemia is caused as a result of hyperactivation of β-cells. High secretion of insulin increases antigen-presenting activity of β-cells and makes them prone to cytokine-induced cytotoxicity [36]. It has been proposed that obesity resulting from high intake of fats and changes in adipose tissue is related to inflammatory gut disorders [37]. The liver is a key to synthesis and metabolism of proteins, carbohydrates, and fats. It is the primary organ responsible for detoxification. Therefore, a balanced healthy diet can help preserve liver homeostasis. In addition, due to the role of liver in glycogen storage, people with autoimmune hepatitis experience insufficient energy intake and thus have to compensate it with more carbohydrate calories [38]. Moreover, it has been shown that high caloric diets exacerbate experimental autoimmune encephalomyelitis (EAE) that is an animal model for MS [30].

The microbial community of the gut plays a key role in digestion and absorption of nutrients. It also affects the gut immunity by providing the gut with immune tolerance against microbial antigens derived from the host microflora. High fat intake and obesity might alter the microbial composition of the gut resulting in aberrant immune responses that can provoke inflammation and autoreactivity in the gut [39]. In this manner, the balance between Th17 and Treg responses which is controlled by the microbial composition of the gut will be subject to change dependent on dietary factors [40, 41].

Fats

Evidence demonstrates that lipid intake can have profound effects on autoimmunity. However, the impact of dietary fatty acids depends on the type and amount of fatty acids fed. A nutrient which has been shown very efficient in averting and curing autoimmune disorders is omega-3 fatty acid. This nutrient prevents inflammation through

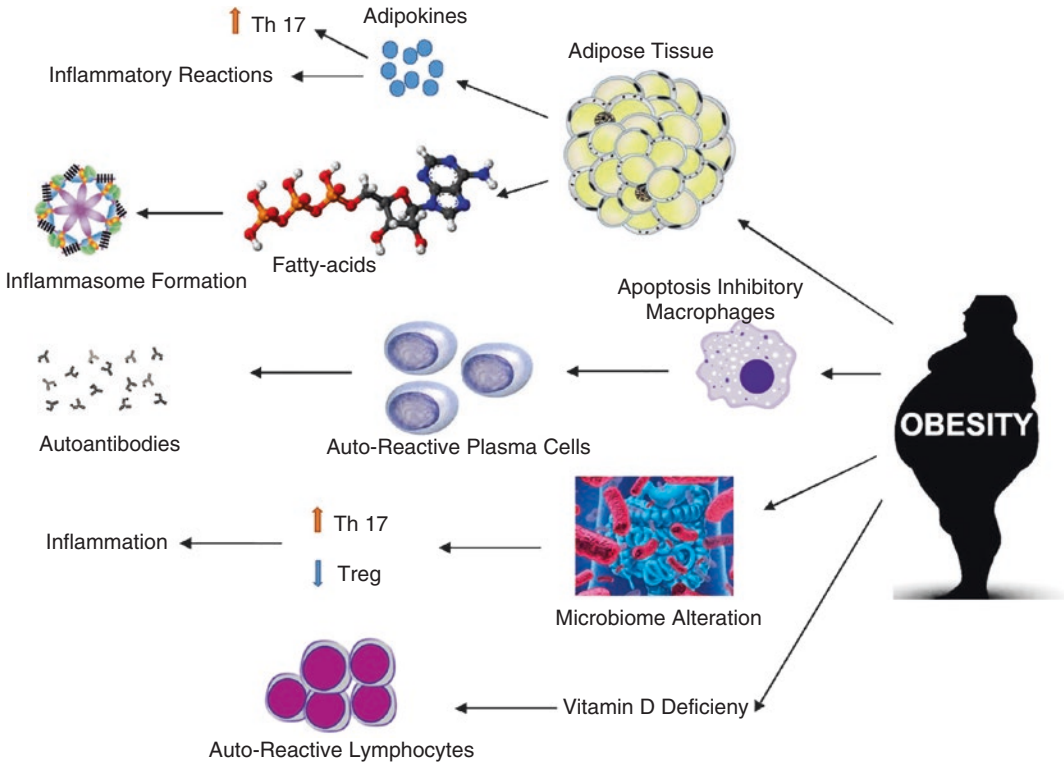


Fig. 21.2 The role of obesity in triggering autoimmunity. Adipose tissue increases immune cells activity and inhibits immune regulatory mechanisms; so, it leads to increasing adipokines which trigger inflammation (1). Obesity simulates AIM macrophages, which increase lipolysis and saturated fatty acids. Saturated fatty acids stimulate native immune responses and mediate inflammatory mechanisms by native immune cytokines such as IL-1β and IL-18 (2). Obesity can directly stimulate Th17 cells proliferation (3). Fatty diet consumption alters digestive tract microbiome, and it promotes gastrointestinal autoimmune diseases throughout increasing Th17 responses and decreasing Treg cells in digestive tract (4). Obesity may decrease vitamin D levels. Low levels of vitamin D are related to several autoimmune disorders (5)

restriction of the production of pro-inflammatory cytokines including IFN-γ. In fact, the optimal balance between omega-3 and omega-6 fatty acids is essential to maintain immune homeostasis [42]. The importance of fatty acids to immunity and autoimmunity was first recognized by Peter Medawar and Jiirgen Mertin [43]. It has been found that some fatty acids such as eicosanoids and polyunsaturated fatty acids (PUFA), especially omega-3, can affect the immunoregulatory mechanisms. A diet rich in saturated fats negatively affects phagocytic activity of macrophages and attenuates function of natural killer cells. Omega-3 fatty acids have also been shown to have regulatory effects on T-cell proliferation in response to PHA and ConA. It is interesting to note that fatty acids also control TGF-β and antioxidant enzyme production [44]. Investigation on NZB x NZW F1 mouse revealed that a diet rich in n-3 fatty acid such as fish oil can induce programmed cell death (PCD) pathway in lymphocytes to successfully suppress autoreactive lymphocytes [45]. Evidence shows that a diet containing high amounts of saturated fats increases IgM thymocytotoxic autoreactive antibodies and thereby the susceptibility to autoimmune diseases [46].

Diets low in fat, particularly essential fatty acids, or high in omega-3 fatty acids from fish oils increase survival and reduce disease severity in animal models. However, eating a diets high in saturated fatty acids (such as linoleic acid) has

been associated with more sever autoimmune disease. Diets deficient in essential fatty acids or diets supplemented with omega-3 fatty acids appear to exacerbate disease. Instead, omega-6 polyunsaturated fatty acids prevent or reduce the severity of autoimmune diseases. Suppression of autoantibody production and T-cell proliferation would stimulate apoptosis in autoreactive lymphocytes and reduce production of pro-inflammatory cytokines. In this manner, omega-3 fatty acids are able to ameliorate autoimmune disease. However, long-term effects of high-dose essential fatty acids might be undesirable as they compromise the body's immune system. The protective mechanisms of omega-6 fatty acids in T-cell-mediated autoimmune disease are less clear but may be related to immunoregulatory effects of circulatory linolenic acid metabolites (dihomo-gamma-linolenic acid and arachidonic acid). It is important to know that linoleic acid does not exactly show the functions of linolenic acid metabolites. In addition, the endogenous rate of linoleic acid conversion into arachidonic acid is slow.

Fatty acids may affect autoimmune disease progression through different mechanisms involving the regulation of gene expression, signal transduction pathways, production of eicosanoids and cytokines, and antioxidant enzyme functions.

Experimental studies revealed that eating diets rich in omega-3 polyunsaturated fatty acids (fish oil) would improve survival in animal models with SLE [47]. Studies have shown that fish oil can relieve tender joints and reduce morning stiffness in patients with RA [48]. Studies reveal beneficial effects of essential fatty acids in systemic sclerosis (scleroderma). One rationale for the use of fatty acids is that derivatives of these fatty acids, such as vasoactive prostaglandins, might help to alleviate chronic ischemia-reperfusion injury, a hallmark of scleroderma [49].

Consumption of saturated fat diets stimulates autoreactive B cells to produce more levels of circulating anti-dsDNA antibodies in mice with SLE. Such diets also increase the risk of renal complications and proteinuria. Evidence from biochemical studies indicates that insufficient fatty acid and eicosanoid metabolism could lead to salivary and lacrimal gland atrophy resulting in immunological and cardiovascular defects in experimental animals with Sjögren syndrome. The study of erythrocytes in patients with primary Sjögren syndrome revealed abnormalities in essential fatty acids. Supporting this, controlled clinical trials of supplementation with gamma-linolenic acid (GLA) as evening primrose oil (Efamol) showed promising results in patients with both primary Sjögren syndrome and systemic sclerosis [50].

Diet enrichment with eicosapentaenoic acid (EPA), a component of fish oil, significantly delayed the onset of crescentic glomerulonephritis and prolonged the overall survival in animal models with antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis. Anti-inflammatory lipid mediators and their precursors were found in the kidney, plasma, spleen, and lungs of the EPA-treated mice. Furthermore, there was a decrease in ANCA titers and an increase in $Foxp3^+$ regulatory T cells in the kidney lymph nodes of the EPA-treated mice [51].

Evidence suggests that decreased consumption of saturated fats and instead increased consumption of vegetables containing omega-3 and omega-6 fatty acids may slow the progression of MS [52]. Additionally, consuming PUFAs and low caloric diets, including fruits, vegetable, and plant fibers, decreases the risk of MS [29]. In contrast, a diet rich in saturated animal fats, including meat and milk, may increase the risk of MS. Consistently, PUFAs have been proposed to play a protective role in Crohn's disease and ulcerative colitis, whereas a diet rich in animal proteins can stimulate inflammatory reactions in the gut and cause IBD-like conditions [53]. PUFAs modulate the gut microbiota through affecting the bacterial composition. PUFAs enhance the proliferation and differentiation of CD4 and CD25 regulatory T cells and decrease the production of inflammatory cytokines including IL17, IFN-γ, and IL-6 in mice. Those mechanisms act against oxidative stress and regulate the production of reactive oxygen species (ROS) in the gut [54]. With a higher content of fat in comparison with plants, animal proteins have been

shown to induce a pro-inflammatory state and increase the risk of dysbiosis and imbalance in the gut microbiota. In this manner, a fat-rich diet may aggravate IBD.

Given that omega-6 and omega-3 fatty acids are not synthesized by the body, there should be an external source providing these fatty acids. As the omega-3 has been related to the downregulation of pro-inflammatory cytokines, the fish oil, which is rich in this essential fatty acid, appears a good nutritional source to control autoimmune liver diseases (ALD). Study of mice demonstrated that a diet rich in omega-3 fatty acids could reduce vascular dysfunction, biochemical hepatitis, and inflammatory activity of Kupffer cells [55]. Consuming a diet rich in n-3 fatty acids like fish oil significantly prevented the relapse of Crohn's disease.

Interestingly, the protective effects of diets rich in γ-linoleic acid derived from fungi or plants have been documented in animal model of MS (EAE). The effects were accompanied by conversion of γ-linolenic acid into the longer-chain n-6 eicosanoid precursor fatty acids, including dihomo-γ-linoleic and arachidonic acids. In fact, γ-linolenic acid was able to predominantly avert both the acute and recurrence phases of the disease. There was also an increase in levels of TGF-β and PGE2 following eating a high-γ-linoleic acid diet. Both TGF-β and PGE2 are known to have anti-inflammatory effects. More precisely, TGF-β can restrain the effector T-cell activity, and PGE2 can prevent the production of pro-inflammatory cytokines by Th1 cells [56]. This clearly explains the protective effect of γ-linolenic in MS [57]. Moreover, the protective effects of linoleic acid against EAE are attributed to its antioxidant activity in which antioxidant enzyme (such as superoxide dismutase) activity is elevated [57].

Sunflower oil, which is a good source of linoleic acid, has shown beneficial effects on MS. In addition, study of guinea pig showed that diets containing high amounts of n-6 and n-3 fatty acids could diminish susceptibility to EAE [58]. Furthermore, a diet high in n-3 and n-6 fatty acids and antioxidant nutrients and low in saturated fatty acids might effectively increase survival and reduce the risk of multiple sclerosis through induction of apoptosis in autoreactive lymphocytes and reduction of pro-inflammatory cytokine production.

Proteins

Proteins in various foods can be resembled to self-proteins and those have been mislabeled. This mislabeling is known as molecular mimicry. Molecular mimicry has been implicated in the pathogenesis of autoimmune diseases especially tissue-specific autoimmune disorders such as multiple sclerosis, spondyloarthropathies, autoimmune thyroiditis, and T1D (Fig. 21.3). This phenomenon is especially seen in T-cell-related autoimmunity. Amino acids are the main structural components of antigens (both self and non-self) involved in peptide-MHC class II interactions. Therefore, amino acids that have similar chemical structure are able to bind at the same MHC class II peptide-binding groove, thereby leading to the stimulation of T-cell responses in a similar way.

One of the best examples of relationship between autoimmunity and molecular mimicry has been shown through cow's milk-feeding neonates. About 40% of patients with T1D have been shown to develop autoantibodies against β-casein (the most abundant protein in cow's milk). Some amino acid sequence homologies are shared between bovine β-casein and peptides expressed by pancreatic β-cells. In about 50% of patients with recent onset autoimmune diabetes, lymphocytes showed an enhanced proliferative response to β-casein [59]. These autoimmune responses may react against insulin-producing β-cells and lead to hyperglycemia. In this manner, exposure to cow's protein provokes the immune system and might cause diabetes. IgA production against β-lactoglobulin and cow's milk protein has been associated with an elevated risk of T1D. The higher proliferation of β-cells was also found among breastfed children [60]. As the breast milk includes high amounts of insulin, regulatory cells will develop because of insulin encounter and promote immune tolerance that prevents T1D. Another example is RA. There is an evidence that some of

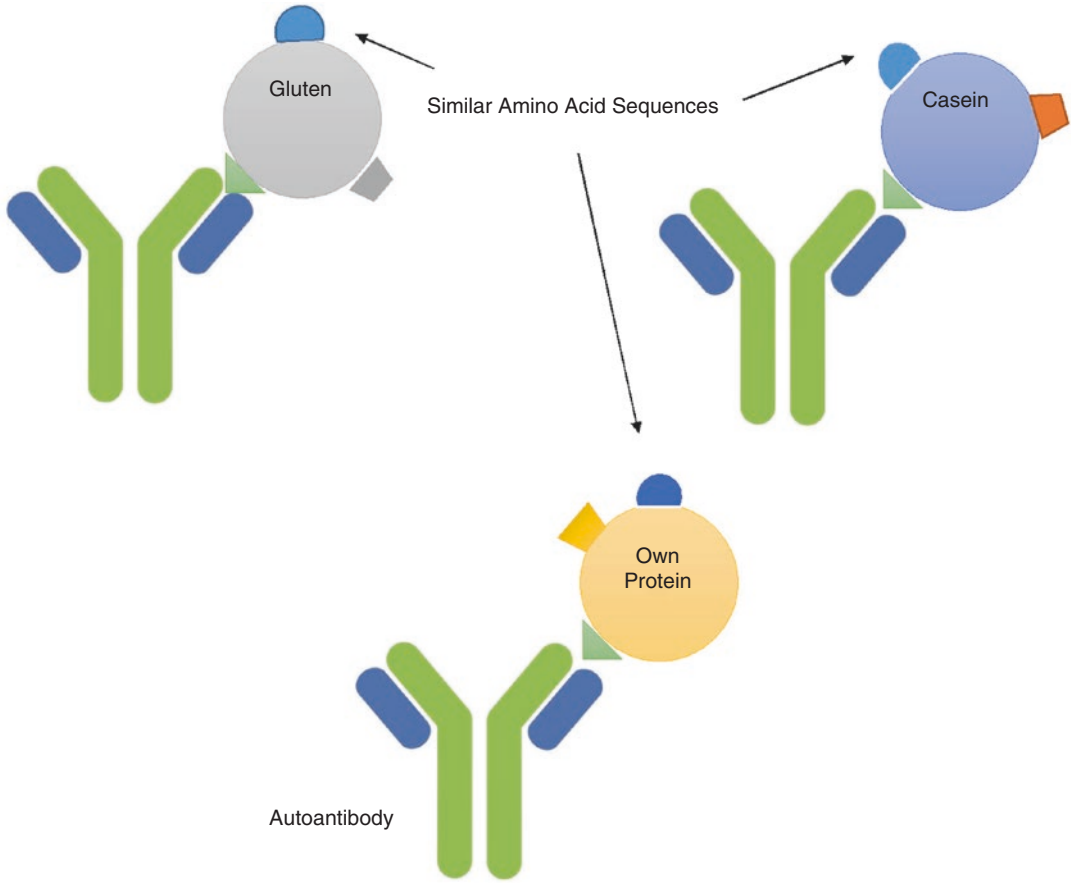


Fig. 21.3 Food proteins can trigger autoimmune reactions by molecular mimicry. Antibodies bind to the specific protein sequences of antigens. Gluten, casein, and self-antigens (such as thyroid antigens) share the same protein sequences. Immune system is not able to distinguish between these molecules, which leads to autoimmunity

the alcohol-soluble proteins from farinaceous grains that serve as a daily staple have potency of molecular mimicry leading to RA.

Almost any protein can cause adverse immune reactions. However, the best-known foods associated with inflammation are:

- Processed meat including hamburger, sausage, bacon, hotdogs, salami, and corned beef
- Milk proteins (especially casein and lactalbumin) in dairy products
- Chicken protein including the light meat and dark meat as well as egg whites
- Wheat protein including breads, pastas, and wheat cereals
- Beef and other red meats
- Soy protein including tofu and tempeh
- Corn protein

It has been proved that high protein intake is related to kidney damage in experimental models of SLE. However, the effect of proteins on autoimmunity depends on the type and molecular structure. Low-protein diets containing high level of aromatic amino acids (tyrosine and phenylalanine) available in beef and dairy products can have benefits for SLE mice. On the other hand, patients with discoid lupus erythematosus (a form of lupus with skin manifestations) report high levels of tryptophan (another aromatic amino acid) in urine. It is possible that tryptophan breakdown and its metabolites act as autoantigens in systemic autoimmunity. Overall, experimental studies suggest that low-protein diets increase life span in animal model with SADs. Human trials demonstrated that the titers of circulating autoantibodies were significantly

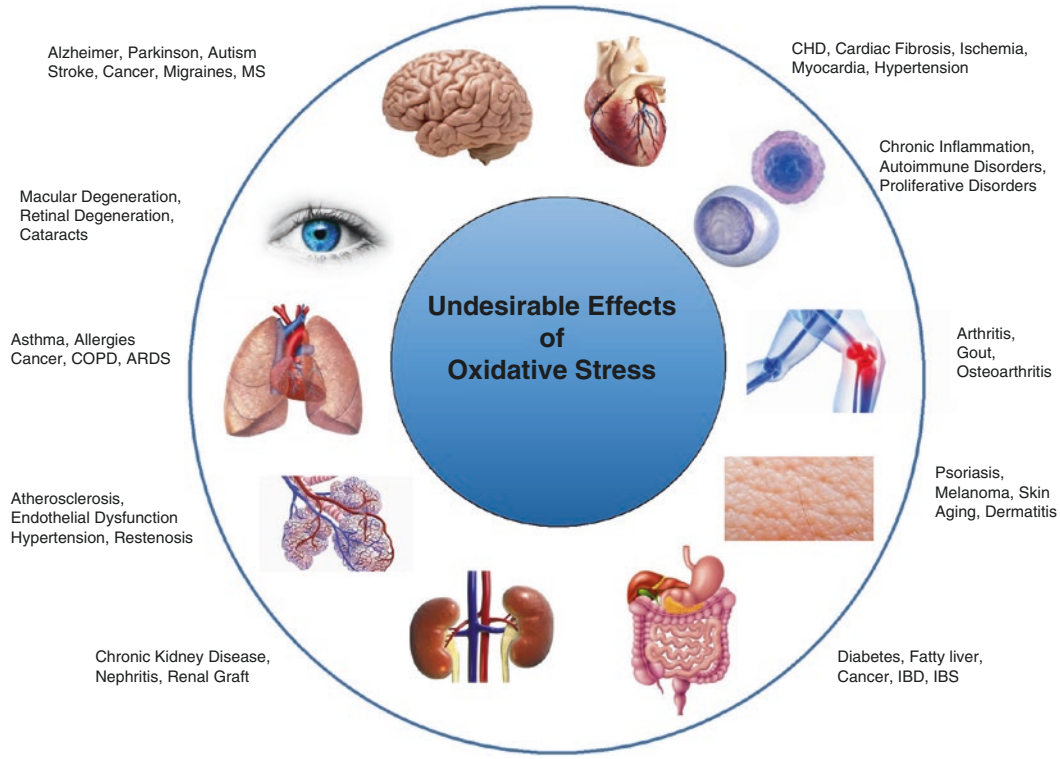


Fig. 21.4 Effects of free radical and oxidative stress on different organs and tissues that result in chronic disorders

decreased in vegetarians, who use protein-free diets [61].

Cereal proteins, gluten, and gliadin were introduced into the permeable lamina propria, and then, antibodies against them induced autoimmune conditions including celiac disease (CD). Thus, a gluten-free diet is the only means of controlling mucosal and intestinal destruction. Actually, intestinal damage significantly reduces the absorption of vital nutrients and minerals. Thus, a gluten-free diet helps the gut to be healed [62]. Like celiac disease, gluten-rich foods may trigger autoimmune responses in some cases with RA. Supporting this, autoantibodies against gliadin (a main component of gluten) were detected in patients with RA. Since antithyroid antibodies are also found in people with celiac disease, a gluten-free diet (such as fruits, vegetables, eggs, corn, beans, nuts, seeds, fish, and poultry) can help to improve intestinal health and vitamin uptake and to lower production of autoantibodies [63]. Studies have found a relationship between wheat gluten and T1D so that increased levels of antigliadin IgG antibodies can be found in people with T1D. Thus, a gluten-free diet can be effective in controlling T1D [64].

Antioxidants

Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defenses. It is discussed in relation with tissue damage in different contexts including autoimmune disorders (Fig. 21.4). The best-documented free radicals are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These highly ROS are capable of damaging nucleus and biologically cell membrane relevant molecules such as DNA, proteins, carbohydrates, and lipids.

Active lymphocytes may cause excessive production of free radicals, potentially leading to apoptosis, necrosis, and consequently cell destruc-

tion. In other words, active T cells and B cells participate in the pathogenesis of autoimmune diseases by the production of autoantibodies, cytokines, and free radicals under the influence of environmental and genetic factors. Oxidative stress is a contributing factor to autoimmune diseases such as SLE, RA, vasculitis, systemic sclerosis, inflammatory myopathies, T1D, MS, and Hashimato's thyroiditis. Free radicals can trigger the immune system by the production of pro-inflammatory cytokines such as TNF-α, IL-8, IL-9, IL-3, IL-6, and IFN-γ. Pro-inflammatory cytokines increase the expression of adhesion molecules, including E-selectin and VCAM-1, and elicit formation of the inducible form of nitric oxide (iNO). These events direct the immune system toward a pro-inflammatory phenotype and finally result in tissue and organ damage. Subsequently, this cascade from injury to inflammation might lead to the development of autoimmune diseases [65]. Given the detrimental role of ROS, consuming a diet low in ROS would be of potential benefit to people with autoimmune diseases.

Antioxidants are low-molecular-weight molecules stable enough to provide an electron for free radicals and neutralize them. Antioxidants can interact with free radicals and terminate the chain reaction before molecular damage occurs. Some antioxidants, including super oxide dismutase, glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body. Other lighter antioxidants (micronutrient antioxidants) are not produced by the body and must be supplied in the diet. The main micronutrient antioxidants are selenium, vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotenes.

A diet rich in vitamins with antioxidant activity (vitamins A, E, and C) can efficiently prevent the destructive effect of ROS by the downregulation of NF-kB [66–68].

Vitamin E supplements can decrease lipid peroxidation in patients with inflammatory disorders. Furthermore, treatment by high-dose vitamin E would delay the onset of SLE and extend life span in animal models. Vitamin E also hampers the formation of N-nitroso compounds which are toxic to pancreatic β-cells [69].

Vitamin A and its precursor, β-carotene, contribute to decrease autoantibody titer in systemic autoimmune conditions. However, a very high dosage of vitamin A may result in an enhancement of cell-mediated cytotoxicity in patients with SLE. Damaged liver is not able to absorb and exploit fats efficiently. Therefore, fat-soluble vitamins (D, E, K, A) should be taken as supplement. Vitamin deficiency is common among people with autoimmune hepatitis (AIH). However, vitamins E and A are both fat-soluble vitamins, and their intake in large doses can be toxic and cause anemia, headache, dry skin, hair loss, nausea, and bone pain.

Vitamin C is a well-known antioxidant, which is important to protect cells against oxidative stress. Like other antioxidants, vitamin C deficiency may also correlate with the onset and increased severity of systemic autoimmune diseases. Studies showed that low vitamin C intake is associated with disease severity in lupus. Adjuvant treatment with vitamin E and C reduces superoxide production by neutrophils in patients with ANCA vasculitis [69].

Selenium is a micronutrient antioxidant essential to immune function. Selenium deficiency is accompanied by loss of immune competence through which both cell-mediated immunity and humoral immunity may be impaired. This might be related to the fact that the selenium-dependent enzymes, e.g., glutathione peroxidase (GPx) and thioredoxin reductase (TxR), have antioxidative effects. These enzymes decrease the formation of ROS, hydrogen peroxide, lipid, and phospholipid hydroperoxides. With adequate amounts of selenium, the hydroperoxide intermediates of the cyclooxygenase and lipoxygenase pathways are reduced, subsequently leading to lowering of production of pro-inflammatory prostaglandins and leukotrienes. In addition, both GPx and TxR modulate the respiratory burst and reduce the superoxide production.

Selenium is mostly abundant in the thyroid gland. It is essential for the proper functioning of thyroid enzymes. It has been shown that selenium is beneficial for decreasing autoantibody production against thyroid peroxidase [70]. Selenium deficiency, actually, reduces the activity of GPx and the cleavage of peroxide within the thyroid gland. Immune-mediated

symptoms will be relieved by thyroid cell necrosis and macrophage activation. These events play an important role in the development and progression of autoimmune thyroiditis [71, 72]. Seafood such as lobster, crab, and tuna are usually rich in selenium. This key nutrient can also be found in nuts.

Animal studies indicate that selenium supplementation can significantly increase survival time in mice with SLE, especially in cases with low levels of blood GPx. However, no obvious effects of selenium supplementation on autoantibody production were observed [73].

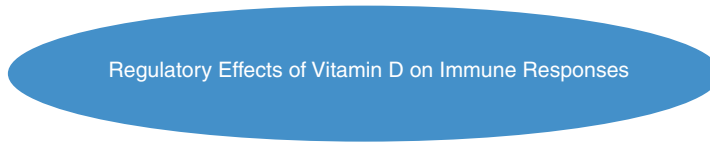
Vitamin D

Since the discovery of vitamin D receptor on the nucleus of immune cells, including APCs, NK cells, and B and T lymphocytes, the effects of vitamin D on the immune system and immune-related disorders became the subject of many immunological investigations. *Regulatory effects of* vitamin D on immune responses are attributed to its ability to induce apoptosis in dendritic cells and chemotaxis and phagocytosis in macrophages while inhibiting the production of T-cell cytokines, proliferation and differentiation of B cells, and antibody production (Fig. 21.5).

Patients with autoimmune diseases frequently report low serum vitamin D. Moreover, disease severity seems to be worse in patients who have vitamin D deficiency. Actually, vitamin D enhances the production of IL-4 and TGF-β while decreasing INF-γ. Experimental studies revealed that supplementation with 1, $25(OH)_2D_3$ (the active form of vitamin D) could prevent the initiation and progression of both systemic and organ-specific autoimmune diseases including EAE, collagen-induced arthritis (CIA) MS, SLE, and RA. The effect of vitamin D on MS is shown in Fig. 21.6.

Because measurements of dietary intake and UV exposure are often based on estimations, evaluating the correlation between vitamin D levels and prevalence of autoimmunity is challenging. Therefore, it might be more useful to analyze the correlation between serum $25(OH)D_3$ levels and autoimmunity.

According to investigations, most of the people with Hashimoto's diseases (approximately 90%) are affected by vitamin D deficiency. However, there is no evidence corroborating the role of vitamin D deficiency as a trigger for Hashimoto's disease. Studies strongly suggest a close relationship between inadequate amounts of this nutrient and autoimmune thyroiditis [74]. As vitamin D is an important nutrient in bone



Innate Immune System		Adaptive Immune System	
Dendritic Cells Maturation	Inhibition	Th1 Cytokine Production (IL-2)	Inhibition
Antigen Presenting	Inhibition	Th17 Cytokine Production (IL-17)	Inhibition
Cytokine Production	Inhibition	B cells Activity and Antibody Production	Inhibition
Antimicrobial Responses (cathelicidin, defensing)	Stimulation	Th2 Cytokine Production (IL-4, IL-5, IL-13)	Stimulation
Pre-inflammatory Cytokine	Stimulation	Treg Activation (IL-10, TGF-β)	Stimulation

Fig. 21.5 Regulatory effects of vitamin D on the immune system

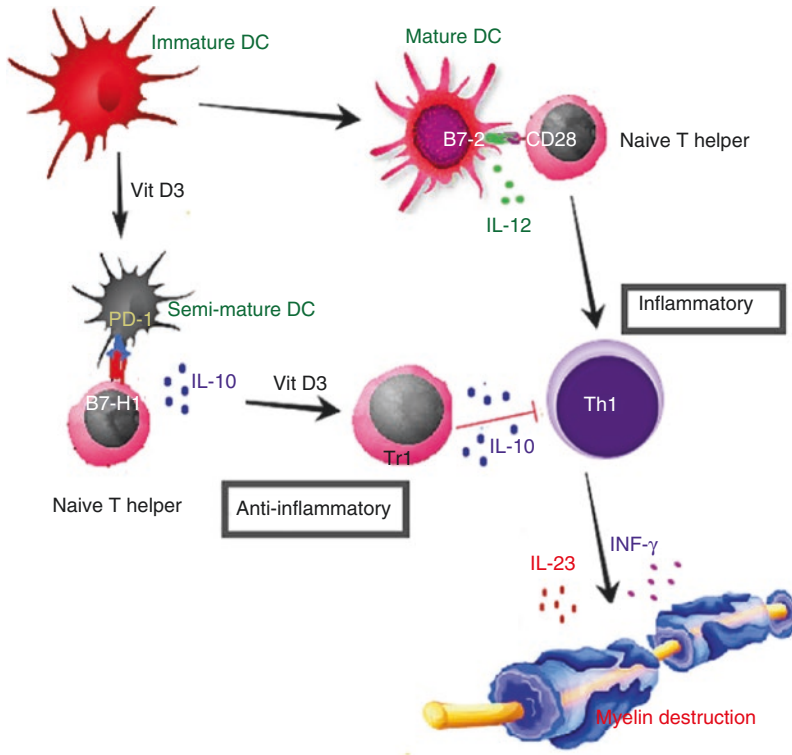


Fig. 21.6 Vitamin D3 promotes differentiation of the immunosuppressive regulatory T cells. Vitamin D3 promotes anti-inflammatory reactions through which semi-mature DCs (smDCs) develop and produce IL-10. IL-10 secretion positively stimulates the expression of B7-H, which is an inhibitory molecule and reacts with PD-1 on the surface of T cells. B7-H/ PD-1 interaction suppresses activation of effector T cells and associated immune responses. It also stimulates regulatory T-cell differentiation, which inhibits myelin destruction. These anti-inflammatory reactions are very important in MS condition

health, higher intake of this vitamin seems an effective strategy for the treatment of hyperthyroidism [75]. It has been shown that vitamin D has the ability for detoxification through enhancing the expression of P450 cytochromes including CYP3A4, CYP2B6, and CYP2C9 [76]. Calcitriol, which is the active form of vitamin D in the body, enhances the expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) on regulatory T cells (Treg) and causes an increase in the Treg frequency. In addition, CTLA-4 and foxp3 negatively control the activation of T cells. Calcitriol also reduces the expression of MHC II molecules on the surface of phagocytic cells, hence resulting in a reduction of autoreactivity [77].

Vitamin D deficiency has been emphasized to be a risk factor for the development of autoimmune rheumatic disorders and persistence of disease activity. Vitamin D deficiency is common among patients with SLE. However, the association between $25(OH)D_3$ level and disease activity is controversial, indicating that there are still many yet unknown exogenous / endogenous factors related to disease activity in SLE. Calcium and vitamin D are not reported to alleviate symptoms of SLE. However, they are recommended as part of the treatment for osteoporosis, which is the most serious side effect of long-term corticosteroid therapy. Moreover, patients with SLE are susceptible to vitamin D deficiency due to the effect of factors such as sunshine avoidance and photosensitivity, chronic renal insufficiency, and the use of medications (glucocorticoids and antimalarial drugs) that may enhance the clearance of vitamin D.

Vitamin D has pleiotropic effects including immunomodulatory, cardioprotective, and anti-

fibrotic effects. Therefore, it is potentially able to modulate the pathogenesis of scleroderma. Vitamin D deficiency is common among patients with scleroderma and is associated with disease activity or phenotype characteristics such as pulmonary hypertension, lung involvement, and extensive cutaneous forms. Low levels of vitamin D correlate with the presence of peripheral neuropathy and lymphoma among patients with scleroderma.

Vitamin D can also interact with insulin-producing cells in pancreas to increase the production of insulin from those cells and therefore decreasing the risk of autoimmune diabetes [78].

Other Vitamins and Minerals

People affected with progressive autoimmune hepatitis face with malabsorption of vitamins (A, D, E, K, B1, B2, B6, B12, and folic acid) and minerals (zinc, iron, calcium, potassium). Thus, diet rich in those vital elements should be taken to help patients with ALD.

Biotin is an important vitamin for the body and especially for the immune system. This vital vitamin is involved in glucose formation, fatty acid synthesis, T-cell proliferation, and antibody production. Considering the significance of biotin in T-cell proliferation and antibody production, consuming the appropriate levels of this nutrient can efficiently promote the immune system function and prevent unwanted reactions. Otherwise, biotin deficiency may affect T-cell proliferation and antibody production that result in the immune system deviation and shift the immune responses from stable protective response into inflammatory pathologic state such as autoimmunity.

Vitamin K is essential to maintain hemostasis. In patients with antiphospholipid syndrome (APS), vitamin K supplement should be taken with caution. Anticoagulants, especially comadines, are used as a primary treatment for a long-time period in patients with APS. In the liver, comadines show an inhibitory activity against vitamin K and their metabolites interfere with the conversion of vitamin K. Whereby, the γ-carboxylation of coagulation factors and their activity are inhibited. Factors associated with unstable control of anticoagulant treatment include the presence of concurrent diseases, drug interactions, genetic factors, and the consumption of vitamin K. Foods with high levels of vitamin K should be consumed sparingly. Because of increased consumption, patients require higher doses to achieve the desired level of anticoagulation. An intake of 60–80 g/day of vitamin K is recommended. Doses above 250 g/day have been associated with an increased risk of thrombosis and embolism [79]. Inversely, very low consumption of vitamin K may result in an increased susceptibility to bleeding. Patients with APS should be advised to maintain a moderate and stable consumption of vitamin K. Food sources with high levels of vitamin K including spinach, kale, Brussels sprouts, broccoli, cabbage, and vegetable oils should be restricted.

Iodine plays an important role in autoimmune thyroiditis and Graves' disease. Actually, iodine incorporates into the composition of thyroid hormones. Therefore, iodine deficiency leads to thyroid dysfunction. This vital nutrient can be found in fish, dairy, and grains. Excess of iodine may cause flare-up and increase production of autoantibodies. Therefore, a precise amount of iodine should be considered as supplement for people with Hashimoto's thyroiditis. Although iodine supplement is of potential benefit to iodine-deficient individuals, it can make the thyroglobulin a more potent antigen for immune cells. Thus iodinated thyroglobulin molecules can act as autoantigens and start a chain of reactions resulting in autoimmunity [80].

Like iodine, zinc is also an essential mineral for thyroid function. Zinc deficiency can induce antithyroid antibodies. Zinc is a nutrient, which is beneficial for preventing autoimmune diseases and has shown instrumental effect in reducing the pathophysiological symptoms of some autoimmune complications like RA and T1D. Actually, zinc can decrease oxidative stress and ROS formation. Hence, using zinc supplementation in addition to foods containing high amounts of fibers and low amounts of simple carbohydrates would improve hyperinsulinemia and hyperglycemia in patients with T1D [81].

Processed Foods

Antigens derived from foods are capable of directly stimulating the immune system and causing autoimmune disorders. Diet manipulation is one of the most accessible ways to improve health and prevent diseases. Throughout history, human lifestyle and diet have changed significantly so that people eat more genetically modified foods these days. Excessive use of antimicrobial substances, antibiotics, heavy metals (arsenic, artificial preservatives, and sweeteners), and salt leads to the development of autoimmune reactions.

Salt intake increases with increased fast food and processed food consumption. High levels of sodium chloride (NaCl) can cause osmotic stress. The osmotic stress can affect the immune system and induce the production of pro-inflammatory cytokines, macrophage activation, and T-cell responses through NFAT5 and p38/MAPK, leading to the initiation of autoreactivity and autoimmune diseases [12]. In vivo investigations showed that excessive consumption of salt is also associated with increased Th17 differentiation. More precisely, high levels of NaCl induce the osmotic stress pathway of serum/glucocorticoid-regulated kinase 1 (SGK1), which is related to Th17 activation. Th17 activation, then, would exacerbate autoimmune conditions (MS and IBD) through the production of pro-inflammatory cytokines and activation of associated inflammatory responses [82]. These events can be simply reversed with decreased salt consumption [83].

Potassium-rich and low-sodium foods including cabbage, potatoes, herbs, tomatoes, spinach, tomato pulp, mushrooms, chanterelles, and fruits (particularly avocado, apricots, bananas, fruit juices, and dried fruit) can efficiently provide the body with nutritional values and decrease the incidence of ALD.

Food processing, particularly by using high temperature, would affect the chemical structure of some nutrients and therefore result in the production of free radicals. As described before, free radicals can react with vital substances in the body including amino acids and sugars. These hazardous compounds can destroy immunoglobulin structure, leading to the development of autoimmune diabetes [84]. N-nitrose compounds, which can be found in processed meat, fish, and beer, are associated with T1D. Nitrate and nitrite have antimicrobial effects and are usually added to foods as preservatives. However, they can be converted into free radicals that are detrimental to the immune system.

Unprocessed foods rich in fibers including vegetables, fruits, legumes, and gluten-free cereals and grains can help the body to combat inflammatory diseases such as celiac disease. Consumption of unprocessed foods would increase the growth of fermentative bacteria such as *Prevotella* and *Butyrivibrio* that engender higher amounts of short chain fatty acids from carboxymethylcellulose, xylane, and xylose. These substances which are rich in unprocessed foods would enhance fermentation and decrease dysbiosis [85].

Ultra-processed food products contain high levels of additives, which affect the body and induce immune responses. For example, manufacturers highly use emulsifiers to attenuate the surface tension and make a more stable emulsion of oil and water. However, this additive can mimic this effect in the body through influencing the hydrophobicity of the phospholipid bilayers and hence increasing the cellular permeability. This may result in introducing new antigens to gut immune cells and promoting inflammation and autoimmunity [86].

Dietary factors have significant influence on microbial flora of the gastrointestinal tract so that different types of bacteria can live in the gut [87]. Some bacterial species of dairy products might be beneficial to immunomodulation. For example, *Lactobacillus casei*, Shirota strain, can provoke antitumor reactions and inhibit the progression of T1D [88]. In mucosal immunity, dendritic cells play a key role in presentation of antigens to the adaptive immune system as well as in activation of regulatory T cells and thereby providing immune tolerance. For that purpose, two important regulatory cytokines (IL-10 and TGF-β) should be present in mucosal environment. Any change in the immune composition, microbiota, and internal environment of the mucosal layer and also excessive contact with

pathogens may lead to inflammation and autoimmunity [89, 90].

Dietary habits and nutritional lifestyle are key elements that affect the microbial composition of the gut. High consumption of processed foods – which contain high levels of saturated fatty acids and salt and are poor in fibers and vitamins – may cause nutritional imbalance and change the normal flora of the gut. The altered intestinal microflora would allow opportunistic pathogens present in the gastrointestinal tract [91]. For instance, it has been shown that a diet rich in fat and gluten can diminish the number of butyrate-producing bacteria. This brings the opportunity for *Bacteroides* to grow and change the gut permeability. Moreover, butyrate reduction results in impaired epithelial integrity and penetration of antigens into lamina propria. All of these events can induce inflammation and immune responses in the gut [92]. Regarding the importance of maintaining microbiota homeostasis, resistant starch that would preserve the gut eubiosis is considered to have anti-inflammatory effect and help to suppress unwanted immune responses in the gut. Resistant starch also increases the butyrate-producing bacteria and hence helps to improve the gut health [93].

Conclusions

The etiology of autoimmune disorders involves environmental and genetic factors. Present diet differs from past and contains more processed foods and saturated fatty acids than before which influence immune system function. In addition, modern diets lack a number of helpful proteins, in particular vitamins and minerals, which can efficiently regulate immune and inflammatory response and therefore control autoimmune conditions. Therefore, the role of nutrition in autoimmune disorders has been of interest.

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22

Immunomodulatory Effects of Flavonoids: Possible Induction of T CD4+ Regulatory Cells Through Suppression of mTOR Pathway Signaling Activity

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Abbreviations

2-DG	2-Deoxyglucose
AD	Atopic dermatitis
AhR	Aryl hydrocarbon receptor
AMPK	AMP-activated, protein kinase
EGF	Epidermal growth factor
FICZ	5,11-Dihydroindolo 3,2 *b*carbazole-6-carboxaldehyde
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
mTORC1&2	Mechanistic target of rapamycin complex 1&2
PTEN	Phosphatase and tensin homolog
T $CD4^+$ or Th	T helper
T $CD8^+$ or Tc	T cytotoxic
TCA	Tricarboxylic acid
Teff	T effector cell
TNF	Tumor necrosis factor
Treg	T regulatory cell

Key Points

- There is body of evidence that flavonoids can modulate immune system. They can decrease production of pro-inflammatory cytokines and antibodies like IgE. Flavonoids also can reduce T-cell activation and proliferation.
- Metabolism of naïve and activated Th cells is totally different, because of their different metabolic demands. Naïve Th cells are metabolically catabolic which increase fatty acids oxidation through AMPK activation, while activated Th cells are anabolic which enhance glycolysis by recruiting PI3K /AKt/mTOR pathway.
- After activation of Th cells, they change their metabolism from catabolism to anabolism which is called metabolic reprogramming. Activated Th cells use glycolysis instead of phosphorylation oxidative in order to supply large amount of energetic demands that is like the same event which happens in tumor cells, whole process called Warburg effect.
- Activation of PI3K/AKt/mTOR pathway plays key role in Th cells differentiation into effector subsets. Activity of mTORC1 promotes Th1 and Th17 differentiation, while mTORC2 induces Th2 differentiation. Suppression of mTORC1 and mTORC2 results in T regulatory induction.
- Studies showed that flavonoids can suppress mTOR in several cancer cell lines.
- Because, during activation of Th cells, they show Warburg effect as same as cancer cells, it can be logical that flavonoids may suppress mTOR, induce T regulatory, and mediate their immunomodulatory effects.

Introduction

Nutrition and metabolism play an important and undeniable role in public health. Although genes have specific importance in susceptibility to diseases, some environmental factors can affect a gene ability to "switch on or off" [1]. In fact, phenotypes are determined by genotypic and environmental factors contribution [2]. Diet is one of the environmental factors that could be considered in the prevention and treatment of several disorders [3], including some autoimmune diseases as MS [3, 4] and type I diabetes [4]. Chemopreventive effects of diet on cancer [5, 6] and autoimmune diseases have also been reported [7, 8].

The immune system plays a critical role in protecting the human body from infectious diseases and cancer. Its two main contributors include innate and acquired immunity responses. The most important feature of innate immunity is its lack of specific recognition. This arm of the immune system responds to all pathogens regardless to their nature [9]. In contrast to innate immunity, acquired immunity recognizes pathogens specifically and responds to each pathogen

according to its nature. Innate immunity is composed of immune and non-immune components, while acquired immunity has only immune elements. The major functions of the acquired immune system rely on immune cells, mainly B and T lymphocytes that recognize pathogens based on their antigenic receptors and respond in different ways. B cells produce antibodies to block pathogen activity and opsonize them for phagocytes. T cells are divided into two subsets: T cytotoxic cells (T $CD8^+$) which kill cancer cells directly and T helper cells (T $CD4^+$) that secrete cytokines and mediators that orchestrate other cells such as B lymphocytes and macrophages [9–11]. Th cells secrete a wide range of cytokines, which can direct the type of antibodies produced by B cells and also are able to activate and polarize monocytes and macrophages. In light of this, Th cells play a central role in the immune system [12].

One of the research areas of immunometabolism is to study the effect of different metabolites on Th cell differentiation. Many researches have focused on the effects of glucose [13], oxygen [14], salt [3], fatty acids [1, 15–17], vitamins [18–20], and amino acids [21, 22] on mechanisms involved in Th cell differentiation. The importance of Th cell differentiation becomes clear when we consider uncontrolled T_{eff} activation against self-antigen that triggers an immune response, through which damages self-tissue and interrupts some organ functions. One of the important dietary factors studied in terms of immunomodulatory effects are polyphenols, and their effects on the level and composition of immunoglobulins, inflammation, and immune cell population content and also their antioxidant effects on cancer cells have been investigated [23–25]. Several studies have reported immunomodulating effects of polyphenols [26, 27]. However, it remains unknown whether Th cells and changes in the ratio of inflammatory/regulatory cells can mediate such effects of polyphenols. Inflammatory and regulatory subsets of Th cells have different metabolic demands. In inflammatory subsets of Th cells, the mTOR pathway is activated. This pathway promotes glycolysis to supply their energy needs. This pathway is inactive in regulatory cells. If the mTOR pathway is suppressed in inflammatory subsets, they differentiate into the regulatory subset. Therefore, activation or suppression of the mTOR pathway determines Th cell differentiation into inflammatory and regulatory subsets (Fig. 22.1). It is still unknown if flavonoids can suppress mTOR function and consequently induce T_{reg} subsets. The current study summarizes the effects of flavonoids on the immune system and subsequently the role of specific pathways like PI3k/Akt/mTOR on immunomodulation and possible effects of polyphenols on this pathway.

Th cell subsets are classified into Th1, Th2, Th17, and T_{reg}. Th1, Th2, and Th17 are effector subsets and trigger immune response to different pathogens [13, 28, 29], while T_{reg} cells restore homeostasis by suppression of T_{eff} cell function after termination of immune response [30, 31]. Precise function of the immune system is important for correct immune response; otherwise two pathological types of responses might occur. On one hand, if the immune system fails to detect pathogens or cancer cells for any reason, the risk of developing disease increases [32]. On the other hand, if the immune system cells by mistake identify self-antigens as foreign agents, the response of killer cells and/or antibody-producing cells interfering with cytokine levels can result in serious damages to the body [33].

Currently, there is only sketchy understanding of the factors and mechanisms involved in autoimmunity. The loss of self-tolerance is one of the important causes of disease. Although the mechanism of loss of immune tolerance is not yet fully understood, some behaviors like smoking may interfere with tolerance and lead to autoimmunity disorders [34]. For this reason, other environmental factors like diet also play a critical role in immune tolerance failure. In normal immune function, both central and peripheral tolerance mechanisms do not allow the immune system to respond against self-antigens [14]. T_{reg} cells are one of these tolerance mechanisms, since T_{reg} cell suppress T_{eff} cells and consequently is able to block unwanted prolonged immune response [35]. Depending on the types of immune cells involved, symptoms and treatment of this disease differ.

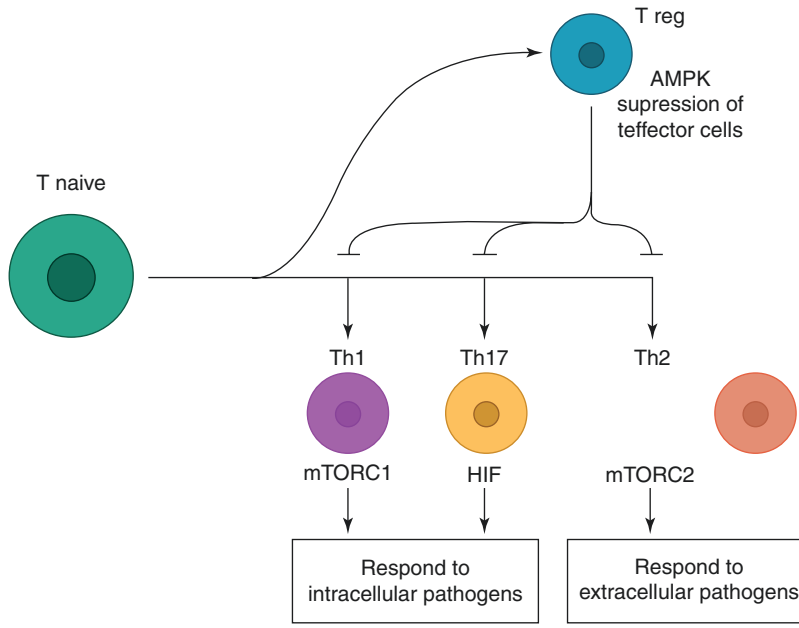

Fig. 22.1 The central role of T lymphocytes in acquired immunity. Acquired immune cells, notably lymphocytes, recognize pathogens specifically and respond to them, according to their nature. T helper lymphocytes secrete various cytokines which are able to activate different immune cells including acquired and innate cells. The various Th subsets rely on different signaling pathways, but all effector subsets upregulate glycolysis. Among differentiated Th cells, T regulatory cells are the only non-glycolytic cells; effector subsets use different signaling molecules; however, all are glycolytic

In addition to the possible role of diet in immune tolerance failure, this question arises whether the immune system and various metabolites can interact with each other. In fact, not only metabolites can affect the immune system, but also the function of the immune system affects metabolic tissues. This mutual interaction is referred to as immunometabolism. Interestingly, malnutrition has a negative impact on immune function. But what is more, oversupply of metabolites is destructive as well [36].

As mentioned above, flavonoids generally have immunomodulatory effects on the immune system. To understand it better, at first we look at the effects of four common flavonoids on the immune system. Here we get general information about the effects of flavonoids on different compartments of the immune system: function of innate and acquired immune cells, antibody production, cytokine secretion, and nuclear transcription factor activity. The entire data suggests that flavonoids can suppress pro-inflammatory immune response.

Flavonoids and Their Effects on the Immune System

Flavonoids are considered as plant secondary metabolites in that numerous pharmacological functions are attributed to them including antioxidant, anti-mutagenic, antibacterial, anti-angiogenic, anti-inflammatory, anti-allergic, enzyme modulating, and anti-cancer [37, 38]. They are defined as phytochemicals which exist either as free aglycones or glycosidic conjugates [39]. Flavonoids are polyphenolic with a wide range of structures [37]. Based on this diversity, they are categorized mainly into flavones, flavanols, isoflavones, flavonols, flavanones, flavanonols and chalcones [39]. The diverse structures of flavonoids have resulted in many properties including anti-cancer and anti-inflammatory effects [37, 39, 40]. Recently, it has been shown that flavonoids can affect immune system response and might have immune-modulator effects.

Quercetin

Quercetin is an abundant polyphenol in nature. It is an aglycone form of a number of other flavonoid glycosides such as rutin and quercitrin which can be found in variety of foods and plants, including apples, berries, Brassica vegetables, capers, grapes, onions, shallots, tea, and tomatoes, as well as different seeds, nuts, flowers, barks, and leaves [41, 42]. Biosynthesis of quercetin starts with phenylalanine in plants [41]. It has been shown that quercetin can affect lipid and glucose metabolisms by reducing oxidative stress and enhancing β-oxidation [43]. In addition, some studies have examined the effects of quercetin on the immune system. In an experimental study, dendritic cells (DCs) obtained from mouse bone marrow were treated by quercetin. This flavonoid could effectively decrease the production of pro-inflammatory cytokines/chemokines and the expression levels of MHC class II and co-stimulatory molecules. These conditions inhibit the LPS-induced activation of DCs. Furthermore, endocytosis of DCs and the LPS-induced DC migration are decreased by quercetin treatment [43]. Quercetin also diminishes Ag-specific T cell activation by reducing the activity of LPS-stimulated DCs [44]. In another experimental study, the effects of quercetin-loaded micro-emulsion (QU-ME) were examined in a model of airway allergic inflammation. Mice received daily oral doses of QU-ME (3 or 10 mg/kg) over the course of 22 days. Compared with control group, QU-ME reduced inflammatory factors including IL-5 and IL-4. However, no change was observed in CCL11, IFN-gamma, and LTB levels. In addition, the nuclear transcription factor kappa B (NF-kappa B) activation, P-selectin expression, and the mucus production in the lung were inhibited by oral treatment of QU-ME [41]. In a study on peripheral blood mononuclear cells (PBMC) isolated from multiple sclerosis (MS) patients and from normal healthy subjects, Sternberg et al. showed that quercetin decreased the proliferation of PBMC and modulated the level of IL-1beta and TNF-alpha released by PBMC in a dose-dependent manner. In this study, the modulation of TNF-alpha increased when quercetin combined with human interferon-beta (IFN-beta) [45]. In another mouse asthma model, Gupta et al. examined the potential of quercetin to relieve asthma aggravation. This study revealed anti-asthmatic potential of quercetin. Treatment with quercetin significantly resulted in a reduction of specific immunoglobulin E (sIgE) production and anaphylaxis signs. Furthermore, quercetin modulated the expression of Th2 cytokines including IL-4 and IL-5. These cytokines play a role in switching IgE class and suppressing the degranulation/secretion of different chemical mediators (PGD2, mMCPT-1 Cys-L, and TSLP) from activated mast cells [46]. Other studies on the effects of quercetin on the immune system showed inhibitory effects of quercetin on cytotoxic lymphocyte function [47], IL-6 production in LPS-stimulated neutrophils [48], and anaphylactic contraction in guinea pig ileum smooth muscle [49]. Moreover, it has been observed that quercetin can regulate leukocyte biology with a stimulus-specific action and affects the balance of Th1/Th2 in a murine model of asthma [50, 51]. Based on these findings, quercetin has a potential role in modulating immune system responses.

Luteolin

Luteolin (3′,4′,5,7-tetrahydroxyflavone) and its glycosylated form luteolin-7-glucoside (L7G) belong to the flavone subclass of flavonoids and are among the most common flavonoids present in aromatic plants and other plant-based foods mostly consumed in the Mediterranean diet. Also, it is well distributed in many medicinal plants and some common fruits and vegetables including green leafy plants such as parsley, sweet peppers, and celery [52–54]. Although glycosylated forms are the most common in nature, it has been reported that luteolin is absorbed in the aglycone form only. Apart from the antioxidant and anticarcinogenic properties, other features as anti-inflammatory and anti-allergic have also been reported for luteolin [55–58]. In an experimental study, treatment of asthmatic models of rats by luteolin

over 8 weeks resulted in a reduction in the total cell count, neutrophil count, eosinophil count, and levels of IL-4 in comparison to a control group [59]. In another mouse study, the effect of luteolin on experimental autoimmune thyroiditis (EAT) showed that luteolin treatment decreased lymphocytic infiltration and follicle destruction in thyroid glands. In addition, luteolin inhibited the interferon-γ-induced increase in cyclooxygenase 2 and the secretion of the pro-inflammatory cytokine tumor necrosis factor-α [60]. In an experimental study on human and murine auto-reactive T cells, Verbeek et al. reported that luteolin was a strong inhibitor for both murine and human T-cell responses. In this study, T-cell proliferation and antigen-specific IFN-gamma production were significantly reduced in response to luteolin treatment. In addition, luteolin appears to be a strong inhibitor of mast cell histamine secretion [61]. Moreover, antibacterial and anti-parasite properties of luteolin have been reported in recent studies [62, 63]. The effects of luteolin on the immune system and inflammation have also been assessed in vivo [64]. Topical application of *Reseda luteola* extract, which is high in luteolin, was as effective as hydrocortisone in decreasing inflammation following skin irradiation with ultraviolet-B light [64]. Overall, it seems that luteolin has beneficial effects on the modulation of immune responses. However, the mechanisms of this action might be variable and are not clearly known. Further studies are needed to shed light on these mechanisms.

Apigenin

Apigenin, or 40,5,7-trihydroxyflavone, is a common dietary flavonoid which is found in many fruits, vegetables, and herbs, such as orange, grapefruits, onion, wheat sprouts, parsley, celery, and chamomile tea [65, 66]. Properties of apigenin include anti-proliferative, anti-cancer antioxidant, and anti-inflammatory activities [67]. Apigenin exhibits anti-tumor effects by decelerating growth and inducing apoptosis through activation of pentose phosphate pathway-mediated NADPH generation in HepG2 human hepatoma cells, induction of apoptosis via the PI3K/AKT and ERK1/2 MAPK pathways, decreasing the viability, adhesion, and migration of cancer cells and modulating angiogenesis and metastasis [68]. The effects of apigenin on the immune system or modulation of immune responses have been assessed in recent studies. In an experimental study, Cardenas et al. reported apigenin significantly modulated NF-κB activity in the lungs. This finding showed the ability of apigenin to exert immune-regulatory activity in an organ-specific manner [69]. In another study on models of rat colitis, administration of apigenin K, a soluble form of apigenin, resulted in reduced inflammation as well as lower colonic damage scores and colonic weight/length ratio [68]. In addition, administration of apigenin K could normalize the expression of some colonic inflammatory markers (e.g., TNF-α, transforming growth factor-β, IL-6, intercellular adhesion molecule 1, or chemokine (C-C motif) ligand 2) [70]. In another experimental study on asthma in mice, Li et al. reported that apigenin administration (5 mg/kg or 10 mg/kg) inhibited OVA-induced increases in eosinophil count and also in Th17 cells. Therefore, apigenin administration might effectively ameliorate the progression of asthma [71]. Furthermore, it has been shown that apigenin in combination with quercetin and luteolin has a protective effect on pancreatic beta-cells injured by cytokines during inflammation [72]. The inhibitory effect of apigenin on mast cell secretion has also been observed in recent studies [51]. Apigenin combined with luteolin is a strong inhibitor for murine and human T-cell responses, in particular auto-reactive T cells [61]. In sum, it seems that apigenin can be considered as a modulator of the immune system.

Fisetin

Fisetin (3, 3′, 4′, 7-tetrahydroxy flavone) is a type of flavonoid commonly found in plants like the smoke tree and numerous types of fruits and vegetables including strawberries, grapes, onions, and cucumbers [51, 73–75]. Some properties of fisetin include anti-cancer, anti-angiogenic, neuroprotective, neurotrophic, antioxidant, anti-inflammatory, anti-proliferative, and apoptotic effects [76]. However, the powerful antioxi-

dant property of fisetin is due to the presence of phenolic hydroxyl group in the flavonoid structure [77]. A few studies have examined the effects of fisetin on the immune system. Song et al. assessed the immunosuppressive effects of fisetin against T-cell activation in vitro and in vivo. Findings of this study showed that fisetin significantly inhibited Th1 and Th2 cytokine production, cell cycle, and the ratio of T $CD4^+/CD8^+$ cells in vitro. Furthermore, fisetin suppressed mouse T lymphocytes through the suppression of nuclear factor kappa B activation and nuclear factor of activated T cells signaling in a dose-dependent manner. The in vivo finding showed that fisetin also inhibited delayed-type hypersensitivity reactions in mice [76]. One study on the effects of fisetin on human mast cells (HMC-1) showed that fisetin could downregulate mast cell activation [73]. In addition, two studies have reported that the anti-asthma properties of fisetin are due to reduction of Th2 response as well as suppression of NF-κB [75, 78]. In an experimental study using a mouse model of atopic dermatitis (AD), Kim et al. investigated the effects of fisetin on AD-like clinical symptoms. They showed that fisetin administration inhibited the infiltration of inflammatory cells including eosinophils, mast cells, and T $CD4^+$ and T $CD8^+$ cells. Furthermore, fisetin was able to suppress the expression of cytokines and chemokines associated with dermal infiltrates in AD-like skin lesions. In a dose-dependent manner, fisetin decreased the T $CD4^+$ cell-induced production of interferon-gamma and interleukin-4 and, in contrast, increased the anti-inflammatory cytokine such as interleukin-10 [79]. Based on these findings, fisetin is able to significantly affect immune system responses.

As mentioned, T $CD4^+$ cells play a central role in orchestrating immune response. Moreover, while regulatory effects of flavonoids on T $CD4^+$ have been observed, the exact mechanisms are under investigation. Here we elaborate why metabolism can play an important role in Th cell fate. What happens to metabolic machinery of Th cells when they get activated? Studies show that metabolic status of naive and activated Th cells is different, because of their different energetic demands.

Metabolism of Th Cells

Resting and naive Th cells don't need great amount of energy. Hence, their metabolic status is generally at baseline. These cells use autophagy and catabolism of fatty acids to supply their housekeeping demands [80]. When these cells are activated, they undergo rapid and excessive clonal expansion. Activated Th cells use anabolism to synthesize different types of essential macromolecules for proliferation, which is highly energetically costly. In fact, activated Th cells switch from catabolism to anabolism, a process known as metabolic reprogramming [80].

The hallmark of Th cell metabolic reprogramming is the use of glycolysis in the presence of sufficient oxygen [81]. If, following activation, Th cells are not able to induce metabolic reprogramming, they become anergic and are not able to respond to pathogens [82]. Therefore, the metabolism of these cells plays a critical role in Th cell activation. However, the main question is: why activated Th cells use glycolysis instead of TCA for ATP production? Why do they prefer to use a low-yield pathway (2 ATP) instead of high-yield cycle (32 ATP)? Although clonal expansion requires energy, it also relies on protein, DNA, and lipid synthesis for cell size augmentation, for which glycolysis provides the energetic drive. Otto Warburg in 1931 found that cancer cells grow in acidic conditions, as they use glycolysis and produce lactic acid (Fig. 22.2).

Below, we discuss how metabolism and immune signals are linked together. Some immunological signals are integrated into metabolic pathways. One of the most important pathways which plays key role in Th cell differentiation is PI3K/Akt/mTOR pathway. The activation status of this pathway is affected by different immunologic signals. PI3K/Akt/mTOR pathway promotes glycolysis, and it is necessary to be increased as it activates glycolysis pathway significantly and also increases the expression of a range of proteins including enzymes and transporters. The PI3K/Akt/mTOR pathway mediates upregulation of glycolysis and prepares cells for proliferation.

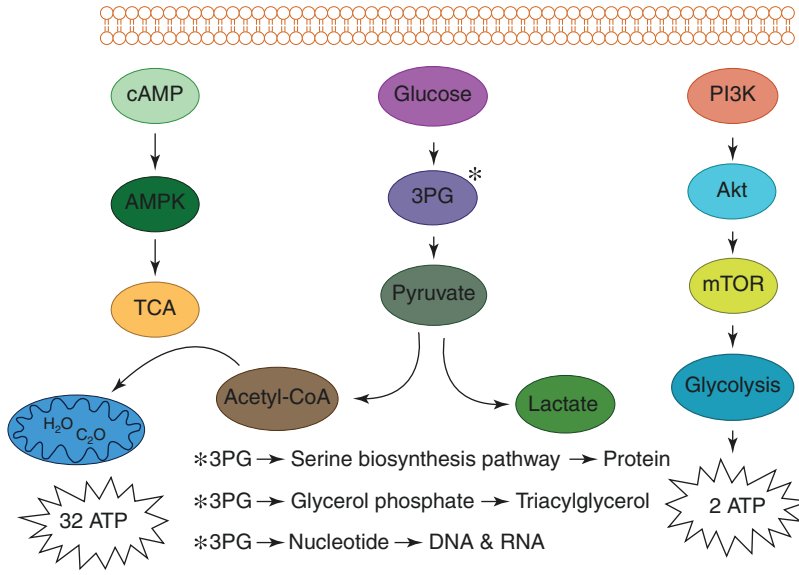

Fig. 22.2 Differences between glycolysis and the tricarboxylic acid cycle. The energy obtained from glucose oxidation and glycolysis is very different, even in oxygen-activated Th cells which use glycolysis. The same situation also is observed in cancer cells. Because of this, the tumor environment is acidic. Activated Th and cancer cells use glycolysis to supply protein, DNA, and lipids to support proliferation

mTOR, Mechanistic Target of Rapamycin

mTOR is a highly conserved molecule in mammalian cells [83], coded as a unique single gene but translated into two different proteins, mTOR complex 1 and 2 (mTORC1 and mTORC2) [82]. These two complexes have different functions. Activation of mTORC1 results in enhancement of translation, cell size, and lipogenesis in white fat tissue, while mTORC2 activation promotes glucose uptake in tissue, enhancement of glucose synthesis, and reduction of gluconeogenesis in liver [83].

Levels of several metabolites (amino acids [83, 84] and glucose), growth factors, energy level (cytosolic AMP-ATP ratio), stress, and immunological signals (CD28, IL-2 [82]) regulate mTOR function (Fig. 22.3) [83]. At the same time, mTOR controls expression of several nutrition transporters [80]. Different cytokines also regulate mTOR activity: IL-7 activates mTOR and inhibits autophagy, IL-4 promotes proliferation through mTOR activation and decrease apoptosis, and IL-12 and IFN-γ also promote continuous mTOR activity [82].

From the immunological point of view, two signals are needed for successful activation of Th cells. The first signal is TCR recognition of antigens and the second is additional signals produced by co-stimulator molecules. If the first signal is not accompanied by the second signal, Th cells will not be able to react (anergy). Anergic T cells are metabolically oxidative. They use oxidative phosphorylation to supply their energy demands [84], and it seems that inhibition of glycolysis is sufficient for induction of anergy. For example, 2-DG, which blocks glycolysis, inhibits Th17 differentiation even under Th17-polarizing conditions [13, 84]. Interestingly, mTOR inhibition using rapamycin promotes the induction of anergy in Th cells, even in the presence of second signal [82, 85]. This phenomenon is explained by the fact that mTOR is downstream of the second signaling pathway; hence its inhibition attenuates the upstream signal.

All reports suggest that mTOR is a mediator in T cells, between immunologic signals and metabolic demands. Further studies show that mTORC1 promotes Th1 and Th17 differentiation and mTORC2 induces Th2 differentiation. Suppression of both complexes results in T_{reg}

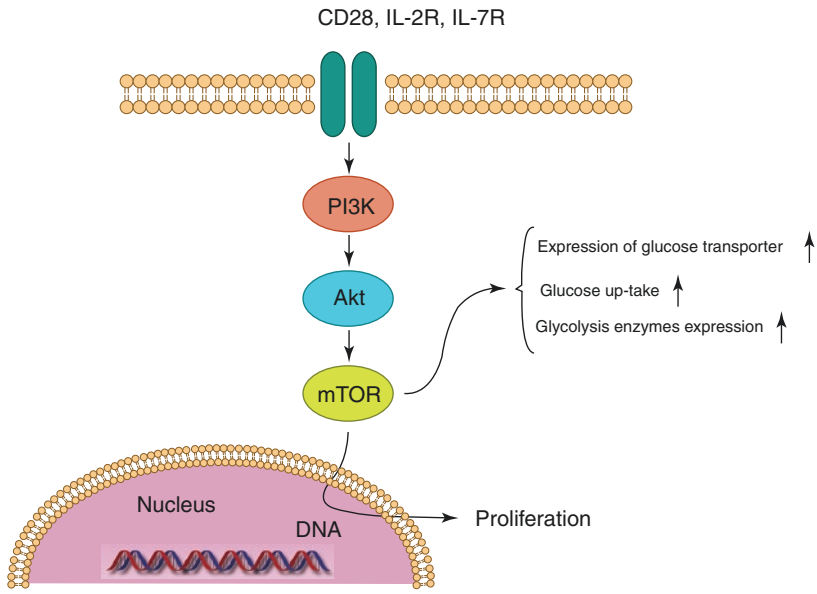

Fig. 22.3 Different signaling pathways are activated by different stimuli. PI3K/Akt/mTOR and AMPK pathways act in contrasting fashion in metabolism and immunity. Several factors like IL-2 and CD28 signaling and growth factors activate the PI3K/Akt/mTOR pathway, resulting in survival and proliferation of different cells. Naïve T cells use lipid β-oxidation to supply their low demands, but after activation, these cells generate a large amount of energy using glycolysis, through changing their metabolic machinery

induction. The effect of rapamycin as an antibiotic and immunosuppressive medicine, which blocks mTOR activation, sheds light on an incredible connection between metabolism and immune function. Earlier studies showed that using rapamycin and knocking out mTORC1 have similar effects [85]. However, the exact mechanism of mTOR in determining Th cell fate is not well understood. It seems that mTOR plays an important role in metabolic reprograming of activated Th cells [21].

In addition to mTOR, the protein kinase AMPK also plays a critical role in metabolism and differentiation of Th cells. Similar to mTOR, AMPK is highly conserved in eukaryotic cells as well [85]. AMPK and mTOR play important roles in metabolism and immunity. Their function in immunity is also against together [15, 86]. By mTOR activation, glucose metabolism is promoted, especially glycolysis, and mTOR suppression by rapamycin results in the suppression of glycolysis and corresponding increase in fatty acid oxidation [15, 36, 85]. Previous studies have shown that induction of AMPK activation has similar results to the suppression of mTOR [15, 36, 85]; by activation of AMPK, consequently fatty acid oxidation is promoted and mTOR function is suppressed. Induction of fatty acid oxidation through mTOR suppression and/or AMPK activation in activated Th cells, as mentioned above, results in T_{reg} differentiation [36, 85].

Rapamycin inhibits mTORC1 and mTORC2 function and induces T_{reg} as well. Although the inhibitory effects of rapamycin on mTORC2 were unclear for several years, Powell and Delgoffe in their investigation in 2010 indicated that specific doses of rapamycin might inhibit mTORC1 and mTORC2 in T cells [82]. They claimed that rapamycin might also promote induction of Treg cells [87].

One of the questions that arises is the possibility of induction of Th subsets in vitro and in vivo through specific metabolites in precise doses by activation and suppression of mTOR. Some previous investigations have shown this effect [88, 89]. These studies help us to understand how our diet influences immune system function. Furthermore, they provide explanations for autoimmune diseases and pos-

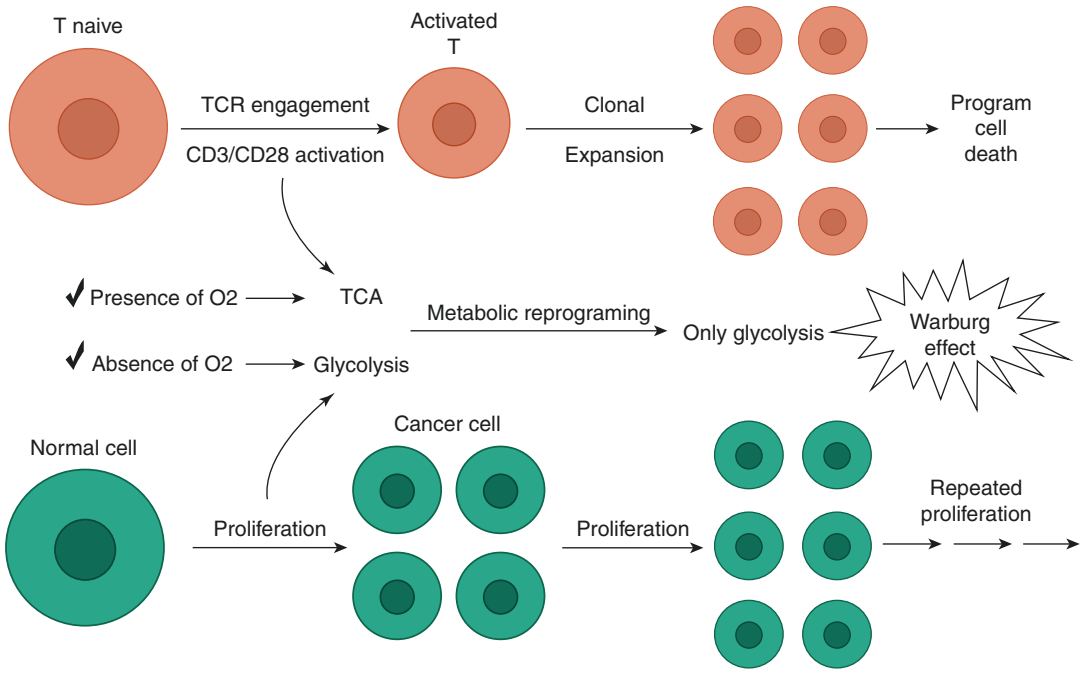

Fig. 22.4 Cancer cells and activated Th cells use similar metabolic programs. Normal and cancer cells use distinct metabolic pathways, because of their different energy demands. Resting and activated Th cells also have different energy demands, because activated Th cells need to growth rapidly. Although cancer and activated Th cells are different, in general, they share a similar feature, their metabolism. The Warburg effect characterizes both cell types

sible key points to treat them. For example, short chain fatty acids are able to induce intestinal T_{reg} in mice. The imbalance of Th17/T_{reg} is responsible for several diseases, like inflammatory bowel diseases (IBD) [16].

Is it possible that accumulation of metabolites in the cells and excessive dietary uptake also modulate mTOR activity? Does this modulation of mTOR activity result in changes in the fate of immune responses? Both answers are yes. Not only specific metabolites like glucose [36, 90], NaCl [3], fatty acids [1, 16, 17, 91], amino acids [21], all-trans retinoic acid [18, 92], cholesterol [93, 94], and vitamins [19] affect mTOR function, but also mTOR regulates expression of metabolite transporters [84]. For example, after Th cell activation, since glucose is the main source of energy for these cells, mTOR activation results in upregulation of glucose transporters. Additionally, the presence of cytosolic leucine and glutamine is essential for mTOR function [21].

The metabolic reprogramming in activated Th cells is the same as that of cancer cells [36, 82]. This similarity may seem logical, as both cells must undergo rapid proliferation, and this process is highly demanding for both energy and metabolic substrates. The difference between Th cell clonal expansion and cancer cell growth is that cancer cells proliferate uncontrollably. Some molecules like mTOR play a crucial role both in the metabolism of cancer cells and activated Th cells, which has been investigated earlier (Fig. 22.4) [82].

Effects of Flavonoids on PI3K/Akt/mTOR Axis Based on Studies in Cancer Cells

As mentioned, cancer cells and activated Th cells have similar metabolisms. Both use glycolysis to supply their demands. Although previous studies about the effects of flavonoids on the immune system might provide some new infor-

Table 22.1 Examples of different flavonoids targeting PI3k/Akt/mTOR pathway in different cell lines

Flavonoid	Duration	Cell line	Effect	Ref
Fisetin	24/48 h	Lung carcinoma cell line	Inhibition of tumor cell growth Increased activation of PTEN, AMPK, and TSC2 Decreased activity of PI3K, Akt, and mTOR	[25]
Fisetin	24/48 h	Prostate cell line	Induction of apoptosis Induction of caspase 3, 8, and 9 activity Decreased activity of cyclins, CDKs, PI3K, Akt	[103]
Gelam honey and ginger	24/48 h	Colon cell line	Inhibition of cell viability	[114]
Pomegranate	24 h	Colon cell line	mTOR, Akt, and PI3k activity suppression and decreased expression	[109]
Curcumin	2 h	Colorectal cell line	Decreased mTOR and Akt expression	[102]
Quercetin	24/48 h	Prostate cell line	Suppression of mTOR and Akt activity	[111]
Baicalein	72 h	Prostate cell line	Induction of apoptosis in cancer cells mTOR and Akt activation decreased	[115]
Butein	24 h	Cervical cell line	Induction of G2/M arrest Induction of caspase 3, 8, and 9 activity Decreased activity of PI3K, Akt, and mTOR	[116]

mation for nutritionists, they are incompletely understood from molecular immunology point of view. In addition, findings from these studies are heterogeneous, for instance, regarding the effect of flavonoids on some inflammatory cytokines like TNF-α [23, 95–97]. Detailed data are available about the impacts of different flavonoids on cancer cell proliferation. Because of metabolic similarity between cancer cells and activated Th cells and lack of sufficient data about flavonoids effects on Th cells or the PI3K/Akt/mTOR axis, here we will focus on results from the cancer field.

Flavonoids also have specific effects on this axis in cancer cells (Table 22.1). Curcumin, a yellow-pigment substance and component of turmeric, significantly increases mTOR suppression and induces apoptosis in renal cancer cells [98]. Curcumin is also able to arrest melanoma cells in G2/M and induce autophagy in these cells. In vitro investigations showed that curcumin inhibits Akt, mTOR, and P70S6K activity. Moreover, curcumin was shown to suppress tumors in BALB/c mice, though this effect was not significant [99]. In breast and prostate cancer cell lines, curcumin inhibits Akt and mTOR function even in the presence of EGF, a ligand of the EGF receptor. The Akt/PI3K/mTOR axis is one of the most important downstream signaling pathways after EGFR activation [100]. The suppression of the Akt/PI3K/mTOR axis, even in the presence of EGF, could be a promising finding in the field of cancer therapy research. Both ligand-dependent and ligand-independent activation of EGFR can cause resistance to current therapies, a major problem in cancer treatment [101]. All results confirm that curcumin induces apoptosis and inhibits tumor cell growth and it is also able to block metastasis [102].

Another attractive polyphenol, fisetin, inhibits mTOR complexes, PI3K [25, 103], and Akt activity in prostate cancer cell lines [25, 103, 104]. It also activates AMPK and PTEN in non-small lung cancer cells [25, 103]. As mentioned before, AMPK and mTOR play contrary roles in metabolism. In cancer cells, AMPK activation and mTOR suppression result in both survival and proliferation failure [105].

Quercetin inhibits the Akt/PI3K/mTOR and Wnt/catenin pathways in lymphoma cells [106]. mTOR inhibition and induction in apoptosis after quercetin treatment in Burkitt's lymphoma cells has been observed [107]. In a cervix cancer cell line, G2/M arrest was observed in the cell cycle.

It also triggers release of cytochrome-C which is an indicator of apoptosis [108].

Other studies have shown that pomegranate polyphenols not only suppress Akt and mTOR expression and function but also reduce IGF expression in colon cancer cells [109]. In 2015 Zhang et al. showed that Aqueous Allspice Extract (AAE), which contains many different flavonoids, was able to activate autophagy signaling in breast cancer cells and induce cell death. AAE acts synergistically with rapamycin and enhances autophagy and cell death. Akt and mTOR signaling are suppressed by AAE [110].

Another similar pathway that is active in tumor cells [111] as well as in Th cells [112] is aryl hydrocarbon receptor (AhR). It plays a central role in the differentiation of Th cells. If AhR is activated by dioxin or kynurenine, T_{reg} cells differentiate in vivo and in vitro, while its other ligand, FICZ, induces the Th17 subset [112]. In prostate tumor cells, AhR shows aberrant expression and its deletion or inhibition results in the inhibition of tumorigenesis and tumor growth. By suppression of AhR, G0/G1 cell cycle arrest occurs in prostate cancer cells. It can be concluded that AhR is necessary for induction of cell cycle arrest and apoptosis by quercetin in prostate cell line [111]. However, the exact mechanism of involvement of AhR in cancer cell apoptosis mediated by quercetin is not well understood yet. Previous studies show that AhR can activate the Akt/PI3K/mTOR pathway, and AhR inhibition results in low PI3K activity and also restores sensitivity to apoptosis in the mouse hepatoma cell line [113].

Because of similar metabolisms in active Th cells and cancer cells, described in detail above, it is expected that the polyphenols can suppress mTOR activity in Th cells. Hence, it can be concluded that polyphenols also induce T_{reg} cells, and these differentiated regulatory cells suppress unwanted immune response against self-antigens.

Conclusion

Before activation of naïve Th cells, they are catabolic. However, after activation and differentiation into effector subsets, they become anabolic. If T effector cells are not able to change their metabolic status, they will be unable to respond to pathogens. The PI3k/Akt/mTOR pathway is upregulated after Th activation, and its suppression results in anergy. By considering the important role of metabolism in the differentiation of Th cells, it seems reasonable that accumulation of specific metabolites may induce Th subsets. Indeed, flavonoids have been investigated for their effects on immune system. Flavonoids are able to modulate immune response, though the exact molecular mechanisms involved in these changes are not well understood. Flavonoids also have anti-proliferative effects on cancer cells through suppression of the PI3k/Akt/mTOR pathway in these cells. As cancer cells and activated Th cells use glycolysis and the PI3k/ Akt/ mTOR pathway plays a crucial role in both cells, it can be concluded that flavonoids also suppress this pathway in Th cells. By suppression of the PI3k/ AKT/ mTOR pathway, T effector differentiation is reduced and T regulatory cells are induced.

Conflicts of Interest Authors declared no personal or financial conflicts of interest.

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Glucan and Its Role in Immunonutrition

23

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Contents

Key Points

- Proper nutrition offers effective, simple, and cheap way to decrease the problems associated with many diseases.
- True concept of immunonutrition is to modulate the immune system and to reach its optimal functions via interventions with specific nutrients.
- Nutrition fortification is a process of adding essential nutrients to food that should contain them naturally. Fortification of a diet with soluble fibers can serve as an example.
- Selenium is a potent micronutrient involved in various facets of mammalian health, including the optimal immune response.
- Numerous studies demonstrated that glucan can stimulate immune reactions in a wide variety of other species, including earthworms, bees, shrimps, fish, chicken, rats, rabbits, guinea pigs, dogs, sheep, goats, pigs, cattle, horses, monkeys, and humans.

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Introduction

People recognized the importance of immunonutrition way before the name itself appeared. Medicine based on prescribing special diets can be found in an essay "On the Old Medicine." Aulus Cornelius Celsus suggested using a salt-free diet for kidney diseases, liver for treatment of night blindness, and milk in case of poisoning. The physician of Marcus Aurelius, Galen of Pergamum, recommended the treatment of various diseases by the specific diets [1]. Much later, Lind used Old Dutch knowledge based on the effects of citrus fruits used by the sailors on their long voyages and prepared one of the first large-scale clinical trials. In his manuscript entitled "On the Most Effectual Means of Preserving the Health of Seamen," he suggested to the British Navy the possibility to eradicate scurvy using fresh citrus fruit. His recommendation was finally accepted and implemented in 1795 and scurvy disappeared. Approximately 80 years ago, the importance of vitamins as essential nutrients was finally documented. It is now ideally accepted that an inadequate vitamin intake can cause severe immunodeficiency.

Proper nutrition offers effective, simple, and cheap way to decrease the problems associated with many diseases. Modern science helps to improve the health of current and future generations. Immunonutrition, best defined as the effect of nutrients on the immune function, represents an important part of nutrition and overall health.

The concept of immunonutrition first appeared 50 years ago when immunology started to separate itself from microbiology. Research in immunonutrition was mainly focused on finding the best nutritional composition that can be used in prevention of infectious diseases and as a support for the treatment of diseases. Gradually, numerous experts started to understand that every infectious disease involves food intake reduction, which subsequently results in changes of the immune system.

Nutrition, appropriate in composition and amount, is one of the basic elements determining human health. Adequate nutrition is strongly involved in our health, including prevention and therapy. Therefore, for more than 50 years, there is intense research devoted to optimization of the diet in order to fulfill nutritional needs at various circumstances of human life. In this chapter, we discuss the basic principles of immunonutrition with particular attention on beta-glucan.

True concept of immunonutrition is to modulate the immune system and to reach its optimal functions via interventions with specific nutrients. Recent development in the study of human microbiome and the comparison between animals reared in germ-free conditions compared to regularly reared animals have underlined the impact of intestinal microflora in modulating systemic immunity. The alimentation can have impact on the bacterial balance in the digestive tract and can consequently influence the intestinal homeostasis, the mucosal immunity, and the systemic immunity [2–4].

Lately, immunomodulatory mechanisms have been studied in cases of critically ill and/or surgical patients. The main reason why immunonuritional rules were applied in these particular problems is the fact that these patients often require an additional supply of vitamins and minerals. This narrow focus, however, overlooks that optimal nutrition is necessary for all individuals. Immunonutrition is focused on these main targets – systemic inflammation, mucosal barrier functions, and cellular branch of immune reactions. Both clinical trials using single immunonutrient and trials using various nutritional compositions have been successful. Addition of glutamine resulting in decreased sepsis found in premature neonates or critically ill patients can serve as an example.

Many of the protective functions of immune cells depend on membrane fluidity which is an important feature allowing immune cells to fully function. As lack of antioxidants can change this fluidity, antioxidants are particularly important for the optimal state of immune system. After decades of research, it is clear that nutritional deficiency is reflected by an inadequate function of immune system, including all facets of both cellular and humoral branch of the immune system (e.g., phagocytosis, cytokine and antibody production, and anti-infection immunity). The question of oxidants and antioxidants is compli-

cated, as oxidants represent the first line of defense, but at the same time, they have to be balanced with antioxidant factors. This is necessary for maintaining optimal health, avoiding prolonged oxidant activities that induce damage on healthy tissues. Therefore, an optimal intake of components with anti-oxidative properties is important. At the same time, it is also important to note that whereas antioxidants work well in experiments, clinical trials trying to establish if antioxidant can help in treatment of numerous diseases almost always failed. Several studies show that antioxidant constituents may be important for development of eye diseases and significantly lower glaucoma risk [5]. The effects of diets on the development of many diseases are well-established. The stroke can serve as an example. A systematic review and meta-analysis of high-quality studies suggested that use of healthy diets (such as Mediterranean or DASH diets) was associated with decreased risk for stroke or stroke-related mortality [6].

The defense system is the body's way of dealing with invading pathogens, non-self material (living or not), and cancer cells. Essential part of these defensive reactions is inflammation, which includes the release of pro-inflammatory cytokines and formation of reactive nitrogen and oxygen species. Depending on how long the inflammation takes place, we can talk about acute or chronic inflammation. The reasons for start of inflammatory reactions are not relevant for the possible long-term implications, which might include an increased risk of cancer. Some nutrients can modulate the immune reactions in such a way that they inhibit inflammatory processes.

Prostate cancer is another example of the role of nutrition. Like with numerous cancers, several hereditary and environmental factors contribute to development of this cancer. However, risk factors are mostly unknown with only race, age, and family history being well-established. Lately, growing proofs emerging from epidemiological surveys and case control studies support the hypothesis that diet plays a crucial role in prostate cancer development. Several nutrients and nutritional supplements have been shown to have beneficial effects including slowing progression of the disease as well as improving conventional treatments. An excellent review of nutritional habits confirmed that consumption of red meat, milk, and fat should be reduced, whereas consumption of fruit and vegetables might have preventive effects [7]. Polyphenols seem to be the molecules responsible for these effects.

Negative changes in lifestyle and most of all in eating habits are known to result in often irreversible changes leading to severe deterioration of numerous health problems. A decrease in the prevalence of traditional infectious diseases has been observed since World War II. One reason might be the progressively more elevated hygienic conditions. The consequent reduction of exposure to microbes and parasites may result in an undereducated immune system. Another problem might be the increased consumption of products composed of highly refined ingredients, because these food products contain significantly fewer micronutrients necessary for immune maturation. The possibility to modulate immune reactions by changes in dietary strategies can be also important for maintaining immune homeostasis. We do not have to ask which food components might impact the immunity, because the answer to this question is simple: they all do.

Nutritional Supplements

Nutrition fortification is a process of adding essential nutrients to food that should contain them naturally. Fortification of a diet with soluble fibers can serve as an example. This particular process stimulates the production of mucus inside the gastrointestinal tract, which subsequently serves as a barrier inhibiting adhesion of pathogens. In addition, presence of debris leads to swelling of intestinal content, acceleration of its passage, and significant decrease of the effect of toxins. It is not surprising that the adequate consumption of fibers represents one of the most potent protective strategies against colorectal cancer [8].

Close relation between nutrition and infections is well-established. This connection is particularly important for young children as they often suffer from both respiratory and intestinal problems. The

effects of protein calorie malnutrition on immune competence are well-described [9]. The subsequent research aimed at addressing how to improve immune functions via nutritional components resulted in the development of commercial food products designed to improve health of both healthy population hospitalized patients. Despite the vast amount of positive results, it is important to keep in mind that we cannot completely save our health by changes in food alone. It is, however, usually better to use thoroughly tested supplements than large quantities of a particular food which is supposed to contain desired component. Resveratrol which might be an important part of so-called Mediterranean diet has been shown to have health benefits. At the same time, we have to understand that the amounts of resveratrol in red wine are so small such that if one wants to obtain a degree of benefit from resveratrol as found in studies using supplements, then he/she should consume several bottles of red wine per day.

The most common nutraceuticals used in general nutrition include arginine (known to increase T cell function), glutamine (acting as an oxidative material for dividing cells), omega-3 fatty acids (reducing formation of pro-inflammatory mediators), monounsaturated fatty acids (known for their ability to reduce cholesterol levels and subsequently risk of cardiovascular diseases as well as for their hypoglycemic effects) [10, 11], nucleotides (with established role in defense reactions), numerous antioxidants (used for prevention of thyroid problems and cardiovascular diseases and for protecting the fetal brain) [12, 13], and glucan. These nutraceuticals are commonly included in our diet. But, clinical trials using exogenous antioxidants failed to observe positive results. Therefore, further investigations are necessary to find the optimal dose and time for successful optimization of immune responsiveness using nutritional approaches.

Zinc

Zinc is a mineral often called “essential trace element” functioning as a catalytic, structural, and regulatory molecule for both enzymes and proteins. It is not surprising that it represents a key trace element in many homeostatic mechanisms of the body. In addition to processes such as immune reaction, zinc is also a critical component of enzymes involved in DNA replication and transcription. The importance of zinc largely arises from its role in the formation of zinc fingers necessary for proper functioning of transcription factors in the regulation of gene expression. This mineral is involved in signal transduction by T cells and B cells as well [14].

Zinc deficiency can cause decreased immune functions. In addition, suboptimal intake of zinc might result in elevated levels of glucocorticoid, depressed thymulin activity, and changes in concentrations of some cytokines. Prolonged zinc deficiency may cause reduction of thymus cells, depletion of T cells, and reduced production of B cell precursors in the bone marrow [15]. Zinc also has a role in tissue remodeling and healing as a catalytic component of matrix metalloproteinases (MMPs). Its deficiency can, therefore, modify MMP production and function in reparation processes and even affect chronic inflammation and bacterial homeostasis in the gut, as it was demonstrated in chronic inflammatory colitis [16]. In older people, low zinc bioavailability was found to reduce resistance to infection. Interestingly, returning to physiological levels for 30–60 days would restore immune function and reduces the incidence of infections (for review see [17]). In this manner, zinc can serve as a nutritional factor, where there is an ample evidence indicating potential consequences associated with its deficiency, but little is understood about the possible effects that a zinc supplement can have.

Selenium

Although toxic in high doses, selenium is an essential micronutrient for animals and humans. It is a well-established nutrient playing an important role in various biological processes, including the optimal immune functioning. Most selenium in the body comes from our diet (e.g., nuts, mushrooms, and seafood). The amount of selenium in an individual food depends on the

area of food cultivation, because the amount of selenium in the soil varies by region.

Selenium was found to have both antioxidant and anti-inflammatory properties [18]. More precisely, it has been shown to increase phagocytic activity of immune cells [19, 20] and improve NK cell function [21]. The effects of selenium on overall health are summarized in recent reviews [22–25]. Most attention on the biological effects of selenium has been focused on cancer. In cancer research, selenium was found to have significant suppressive effects on breast cancer development via epigenetic mechanisms [26–29].

The search is ongoing for missing bioactive molecules that exert synergistic effects in combination with selenium. Of particular interest to the present chapter is that selenium nanoparticles-glucan composites were found to have strong anti-cancer effects [30]. Glucan, particularly when combined with selenium-linked pseudodisaccharide, would significantly reduce carcinogenesis in several mouse cancer models [31]. Studies confirmed that combination of glucan with selenium can significantly improve biological effects of each one [32].

Beta-Glucan (β-Glucan)

During 50 decades of research, countless types of glucan have been described, all under the same name glucan. There are numerous sources for glucan, from yeast to mushrooms and grain. Glucan can be relatively easily isolated from every species of yeast, and the reason for the popularity of *Saccharomyces cerevisiae* is purely based on its availability. Glucans represent a major structural component of the cell wall in fungi and some plants. Different physicochemical parameters (solubility, primary structure, molecular weight, branching, and polymer charge) all play a role in determining whether the polysaccharide modulates immune reactions. Some conclusions can be made. Branched or linear 1,4-β-glucans have very limited activity, if any. Glucans with 1,6 configuration often have limited activity. The highest stimulation of defense reactions has been achieved with β-glucans that have a 1,3 configuration with additional branching at the position 0–6 of the 1–3 linked D-glucose residues. Among all glucans, those with a 1,3 configuration are best characterized in the literature [33].

The original studies investigating the effects of glucan on the immune system were focused on mice. Subsequent studies demonstrated that glucan can stimulate immune reactions in a wide variety of other species, including earthworms, bees, shrimps, fish, chicken, rats, rabbits, guinea pigs, dogs, sheep, goats, pigs, cattle, horses, monkeys, and humans. Glucan can also help protect plants such as turmeric or tobacco [34]. Not surprisingly, it has been concluded that glucan is an immunostimulant active in every biological species and that it is one of the few immunostimulants active across the evolutionary spectrum [35]. For detailed reviews on glucan history and function, see [35–38].

Glucan was first used in clinical setting for the treatment of cancer in 1980 [39]. Western medicine was, and unfortunately still is, more reluctant. However, clinical trials testing glucan supplementation in treatment of various diseases are currently running all around the world.

To fully establish positive stimulation of the immune system by a particular nutritional supplement, precise investigations are necessary. Several clinical trials by our team evaluated the effects of glucan supplementation in children with chronic respiratory problems. Randomized, double-blind, placebo-driven studies were conducted to compare the control (placebo) group with a group consuming food supplemented with glucan for 30 days. There were significant improvements in the production of secretory antibodies (IgM, IgA, and IgG), lysozyme, CRP, and calprotectin. In addition, strong improvements in endurance were observed [40–42]. Data from our clinical trials clearly showed that even short-time food supplementation with a daily use of beta-glucan can have a positive effect on human health.

Glucan as Prebiotics

Little is known about glucan as a probiotic polysaccharide. However, a fish study showed that glucan can act as a probiotic supporting

Lactobacillus activity and thereby reducing mortality from challenge with *Aeromonas* [43]. Additionally, addition of glucan and starch during cold storage strongly increased survival of bifidobacteria strains in yogurt [44]. In addition, glucan with high prebiotic effects was isolated from *Lactobacillus plantarum* [45]. Feeding of glucan as a prebiotic had positive effects both in shrimp and calf models. In both cases, it caused significant improvement in humoral defense parameters [46].

A placebo-controlled clinical study evaluated potential prebiotic activity of glucan in humans. Fifty-two healthy volunteers consumed glucan or placebo for 30 days. The effects were observed only in individuals over 50 years of age, where glucan consumption induced a strong bifidogenic effect and an increase in bifidobacteria levels [47]. Further studies have confirmed these properties of glucans as important prebiotic substrate useful for enhancing the survival and effects of probiotics in medical practice [48, 49].

A more detailed study found that different glucan types isolated from the same source would differentially influence the growth of probiotic bacteria (for review, see [50]). Potentially beneficial effects of glucan on intestinal microflora are already considered good enough to conduct a clinical trial of glucan in polypectomized patients.

Conclusions

With a recent worldwide rise of antibiotic resistance, the World Health Organization (WHO) recommended a cutback on antibiotic prescription and consumption and, at the same time, the development of new therapeutic strategies. In addition, the population of developed countries is getting older and living longer. Therefore, the age-related reduction in the immune system function might be at least partially compensated by the diet based on optimal consumption of nutraceuticals. Among numerous food additives and supplements commercially available, only a limited number have been vigorously tested and evaluated. Beta-glucan, with over 10,000 published independent studies, is by far the most tested and most promising supplement.

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Nutrigenomic Immunity

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Key Points

- Single nucleotide polymorphism (SNP) studies have established the interaction between polymorphisms within the genes related to immune and inflammatory responses (CRP, IL-1, TNFα, IL-6, LTA4, and SCD-1) and diet composition (vitamin D status, botanical formulation, fat intake, and fatty acid supplementation).
- SNP studies also show that the content of inflammatory markers (IL-6, CRP, and α2-microglobulin) would be influenced by the interaction between polymorphisms within metabolic pathways (GCKR, Fok-1, and FADS 1/2) and diet composition (fat intake, nutritional counseling, and vitamin D therapy).
- Human and animal studies demonstrate differential expression of genes related to immune response and inflammatory processes in response to single (acute) or repeated daily (chronic) consumption of different dietary interventions (vegetables, fruits, and other plant-derived products; fish oils and meals; plant oils and meals; micronutrients; ethnic dietary patterns; calorie restriction; oral challenge tests; different protein sources; and nutritional stress).
- Immune-related gene-diet interactions might affect anthropometric parameters, metabolic profile, and cardiovascular measurements and thereby alter individual susceptibility to metabolic (obesity, diabetes, and non-alcoholic fatty liver diseases), autoimmune (Crohn's disease), and cardiovascular disorders (atherosclerosis).

Introduction

With the manifestation of radical advances in genetic tools along with the multiplicity of far-reaching effects of dietary patterns on health and diseases, a new field of science was born: nutrigenomics. The years from 1960 to 2003 recorded sporadic opinions and review articles for the application of omics to the nutritional sciences, while 2004 is the year nutrigenomics successfully energized scientists to pursue and share their knowledge more actively about the exploration of nutrient effects on the cellular and molecular signaling by means of genetic tools [1]. We searched PubMed with the terms "nutrigen∗ AND (immun∗ OR inflammat∗)." The present chapter would provide a synthesis of evidences linking dietary patterns with immune system-related genetic variations and gene expression.

Human Studies

Single Nucleotide Polymorphism (SNP) Studies

SNPs in Genes Related to Immune and Inflammatory Responses

In South Africa, the co-presence of 25-hydroxy vitamin D insufficiency or deficiency (< 75 nmol/L) and CRP elevation (>3 mg/L) was detected in more than 40% of random population sample of women who were healthy and above 30 years of age (n = 660) [2]. This so-called case phenotype was associated with worse cardiovascular and anthropometric measures including higher systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference (WC), hip circumference (HC), weight, and BMI. Among the investigated SNPs in the CRP gene, three (rs3093068, rs3093062, and rs3093058) showed association with an increased risk of case phenotype whereas two (rs2794520 and rs7553007) with a decreased risk of that phenotype.

The possible association of CRP polymorphisms (rs1205, rs1417938, and rs2808630) with plasma content of fatty acids and inflammatory markers was investigated in a sample (n = 262) from the health survey of São Paulo (ISA-2008) [3]. Participants were divided according to whether levels of inflammatory markers fall within inflammatory ranges (INF) or not (NINF). When compared to the NINF group, the

INF group had higher WC, SBP, and DBP and higher plasma concentrations of palmitic acid, TG, SCD-18, hsCRP, TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, adiponectin, and Sicam-1, while they had lower levels of PUFA, n-6, arachidonic acid, and D5D (C20:4/20:3). Both rs1205 and rs1417938 revealed significant interactions with palmitic acid levels. Further the interaction of CRP polymorphisms and inflammatory background (being INF or not) influenced plasma fatty acid levels in several ways.

A clinical trial composed of a 2-week run-in period and a 6-week treatment with n-3 fatty acid supplementation in 191 subjects was depicted to address whether the effect of n-3 fatty acids on the expression of inflammatory genes in PBMCs is modulated by polymorphisms within CRP (rs1800947, rs3093059, rs1130864, and rs1205), IL-1β (rs1143633, rs1143634, rs16944, rs3136558, and rs1143627), IL-6 (rs2069861, rs2069840, rs2069837, rs2069827, and rs1800797), and TNF-LTA cluster (rs1041981, rs2857706, rs1800629, rs2239704, rs3093662, and rs2229094) of genes [4]. Inclusion of n-3 fatty acid supplementation into the diet led to an increase in BMI and in the expression of TNF-α and IL-6 alongside a decrease in TG levels. There were found significant gene-diet interactions between plasma IL-6 content and IL-1β (rs1143627 and rs16944) and IL-6 (rs2069840 and rs1800797) polymorphisms. Polymorphisms within TNF-LTA gene cluster (rs1800629 and rs2229094) were associated with TNF-α and CRP levels as well. Particularly, when compared to those with wild-type homozygous genotype, patients carrying the mutated allele of the rs2229094 had higher plasma TNF-α levels.

A nutrigenetics proof-of-concept study addressed whether the effects of botanical formulation (rose hips, blueberries, blackberries, and grapevine) on increased levels of CRP would be influenced by the IL-1 gene cluster polymorphisms [5]. Healthy adults with elevated serum CRP levels (2–10 mg/L) were genotyped at three loci (IL-1β −511, IL-1β +3954, and IL-1α +4845) and accordingly categorized as IL-1^{Pos} "if they had any of the following three genotypes: (1) were homozygous for the common allele C at IL-1β – 511), (2) carried two copies of the less common allele T at IL-1α +4845), or (3) carried one copy of the less common allele T at IL-1α +4845 plus at least one copy of the less common allele T at IL-1β +3954." After 12-week treatment, the gene expression in PBMC was measured. There were lower levels of IL-1β in the whole group of subjects treated with botanical formulation and that reduction in the expression of IL-1β was more pronounced in IL-1^{Pos} people than IL-1^{Neg} people (61.2% vs. 43.8%). Further interesting was the higher inhibition of IL-1β production in monocyte cell lines treated with plasma from IL-1^{Pos} subjects rather than in those treated with plasma from IL-1^{Neg} subjects (28.2% vs. 13%). Botanical formulation appeared effective on the reduction of elevated CRP levels neither in IL-1^{Pos} subjects nor in IL-1^{Neg} subjects. This might be due to the wide range of response to therapy, because a reduction of CRP > 10% was observed in 55% and 26.3% of IL-1^{Pos} and IL-1^{Neg} subjects, respectively. The corresponding rates for a reduction of CRP > 30% were 40% and 10.5%.

A study [6] of 99 patients with Crohn's disease demonstrated the direct association of disease activity with the TT genotype on the polymorphism TNFα – 857. Further, there was association between disease activity and dietary intake of saturated and monounsaturated fats and high n-6/n-3 PUFA ratio. Interestingly, the latter association was pronounced in patients positive for the polymorphism TNFα – 857.

The study [7] was performed on a sample (n = 707, mean age: 22.7 years) from the TNH study. Participants were genotyped for the polymorphism IL-6 rs7801406. A direct association was found between dietary fat intake and homeostasis model assessment of insulin resistance (HOMA-IR) in subjects with G/G genotype, whereas the inverse of this association was observed among A/A homozygotes.

A clinical study [8] investigated the possible impact that polymorphisms (rs11190480, rs3071, rs3829160, rs2234970, rs10883463, and rs508384) within the gene SCD-1 might have on response (as evaluated by cardiovascular function) to n-3 PUFA supplementation. Participants (n = 210) underwent a run-in period of 2 weeks before the trial of treatment with fish oil capsules

for 6 weeks started. Treatment attenuated plasma parameters such as glucose, TG, palmitoleic acid, SCD 16 indices, oleic acid, and SCD 18 indices. Both pre- and post-treatment plasma concentrations of IL6 were lower among subjects carrying the rs3071 CC genotype. Lower CRP concentrations were correlated with the rs3829160 GG genotype and higher SCD18 indices with the rs2234970 CC genotype as well.

The study [9] based on a sample (n = 3402) of the Strong Heart Family Study (SHFS) addressed whether a six SNP haplotype (rs61937881 (C), rs1978331 (G), rs17677715 (T), rs2660899 (G), rs2540482 (T), and rs2660845 (A)) within the LTA4H gene modulates the relationship of habitual dietary intake over the past 12 months to the carotid ultrasound measurements and atherosclerosis risk factors (BMI, alcohol consumption, hypertension, diabetes, and cigarette smoking). Haplotype carriers did not differ significantly in atherosclerosis measures from non-carriers. However, the interaction of haplotype with dietary intake of n-3 and n-6 fatty acids had a significant impact on the intima-media thickness (IMT) and vascular mass.

Whether the interaction of HLA-DQ2/8 genotypes with gluten dietary intake change plasma protein content was investigated in a sample (n = 1114) of the TNH study [10]. Gluten intake showed correlation with concentrations of several proteins. After adjustment for potential variables, the correlation, however, remained significant only for α2-microglobulin. None of HLA variants did affect the correlation.

Comment

The first series of SNP studies establish the interaction between polymorphisms within the genes related to immune and inflammatory responses (CRP, IL-1, TNFα, IL-6, LTA4, and SCD-1) and diet composition (vitamin D status, botanical formulation, fat intake, and fatty acid supplementation). Consequent changes in the content of inflammatory markers (CRP, IL-6, TNFα, and IL-1β), lipids (triglycerides), and fatty acids (palmitic acid, oleic acid, palmitoleic acid) may contribute to the pathophysiology of vascular and metabolic disorders.

SNPs in Genes Related to Metabolic Pathways

An open-label and single-arm clinical trial showed the effectiveness of 6-month nutritional counseling on anthropometric parameters (BMI, body fat, trunk fat, WC, HC), blood pressure (SBP and DBP), liver imaging, fibrosis score, fasting glucose, HDL, LDL, albumin, HbA1c, CRP, Visfatin, and oxLDL in overweight or obese people with non-alcoholic fatty liver disease (NAFLD) [11]. After stratifying according to the genotype at the glucokinase regulatory (GCKR) rs1260326 polymorphism, only T carriers showed improvement of paraclinical parameters such as endoscopic ultrasound elastography (EUS), fibrosis score, blood concentrations of fasting glucose, total cholesterol, LDL, and HDL, the SGOT/SGPT ratio, HbA1c, and serum CRP.

Through a randomized controlled trial [12], the effect of Fok1 vitamin D receptor gene polymorphism on the response to 12-week vitamin D therapy was evaluated in patients with diabetes (n = 140, age range: 29–67 years). At the end of dietary treatment, 23 patients could not achieve vitamin D sufficiency; among them few (n = 6) had vitamin D deficiency. When stratifying according to the genotype at the Fok1 polymorphism, this trend was confirmed among FF homozygotes and heterozygotes, but not for ff homozygotes; among them there was 1:1 ratio for vitamin D deficiency and insufficiency at the end of trial. Supporting this, patients carrying the FF genotype had significantly higher levels of 25(OH) D than those with ff genotype. Further, the reduction in concentrations of CRP and IL-6 was more pronounced in FF homozygotes compared to that of heterozygotes and ff homozygotes.

A study of a sample (n = 878) of TNH participants [13] investigated the association between six polymorphisms (rs174579, rs174593, rs174626, rs526126, rs968567, and rs17831757) in the FADS1/2 gene cluster and plasma content of CRP and fatty acids. Plasma CRP levels negatively correlated to linoleic acid levels and FADS1 desaturase index whereas positively to dihomo-γ-linolenic acid and the aggregate FADS1/2 desaturase index. Interestingly, all the investigated SNPs showed association with the FADS1 and

the FADS1/2 desaturase indices and arachidonic acid levels. Although not significant after multiple testing correction, the only polymorphism associated with CRP levels was rs526126.

The interaction between polymorphisms within FADS (rs102275 and rs174448) and ELOVL (rs2236212 and rs17606561) genes, fatty acid content in serum and cord blood, and development of pediatric allergic symptoms was investigated in a sample of Swedish birth cohort including a total of 211 subjects (no allergy = 88, atopic eczema = 41, and respiratory allergy = 82) [14]. The minor allele of both FADS polymorphisms was significantly correlated with higher concentrations of dihomo-γ-linolenic acid as well as lower concentrations of arachidonic acid in cord serum. In the adolescent serum, the negative association of rs102275 with arachidonic acid levels remained significant. When correction analyses were applied, none of the investigated SNPs affected susceptibility to pediatric allergy in this cohort.

Comment

The second series of SNP studies show that the interaction between polymorphisms within the genes related to metabolic pathways (GCKR, Fok-1, and FADS 1/2) and diet composition (fat intake, nutritional counseling, and vitamin D therapy) would influence the content of inflammatory markers (IL-6, CRP, and α2-microglobulin), fatty acids (dihomo-γ-linolenic acid and arachidonic acid), and lipid (HDL, LDL, and oxLDL) and sugar profile (fasting glucose and HbA1c). In this manner, these interactions might confer susceptibility to the metabolic disorders particularly diabetes and NAFLD.

Gene Expression Studies

Vegetables, Fruits, and Other Plant-Derived Products

Healthy smokers exhibited a wide range of response to a single dose (5 gram) of curcumin in terms of vascular and endothelial function [15]. The whole-level population analysis indicated no effect related to curcumin. Subgroup analyses, however, identified a female-specific effect of curcumin on the improvement of FMD. Further, individuals who had FRS lower than 16% showed a significant increase in FMD. Consistently, analysis of PBMC indicated no effect of curcumin on gene expression in the whole population, while subgroup analyses demonstrated differential changes in gene expression for men and women separately. In women, the immune system-related genes highly affected by curcumin were related to chemotaxis (CXCR6, CXCR3, CXCL9, CXCL10, CXCL16, CXCL17, CX3CR1, CCR1, CCR7, and CCL3) and inflammation (IL-6 and STAT1), and all of them were upregulated with fold change above two, while a total of only six genes were affected in men; among them were IL-1R2, CCL20, CCL22, CCR5, and CXCL6, and all of them were downregulated.

The study [16] investigated the effect of treatment with a single dose (100 μg/mL) of 90MX: cranberry juice powder or CE: cranberry extract powder) on the gene expression response to LPS in human monocytic cell line THP-1. Ac compared to non-treated cells, LPS accompanied by an increase in the mRNA expression of inflammation-related genes including TNFα, CAT, and SOD1 as well as a reduction in the expression of IL-6 in CE-treated cells. Such anti-inflammatory response was not evident in 90MX-treated cells.

An open, double-blinded, randomized, crossover study allocated 23 overweight otherwise healthy men (mean age: 56 years) to control drink plus placebo, control drink plus hesperidin, and control drink plus orange juice for 4 weeks [17]. Microarray analyses of leukocytes identified three clusters of DEGs: genes differentially expressed in response to orange juice, genes differentially expressed in response to hesperidin, and genes differentially expressed in response to both orange juice and hesperidin. Interestingly, the effect of orange juice was more noticeable in terms of the number of DEGs and the amount of change than hesperidin. The genes whose expression was altered by both orange juice and hesperidin were mainly involved in the signal transduction, cell adhesion, immune response, cell proliferation, chemotaxis, and lipid metabolism.

The study [18] was designed to address the effects of an antioxidant dietary treatment on the gene expression profile in airway samples. Ten adults with asthma (mean age: 63 years) underwent a 2-week antioxidant diet (which contained no more than one piece of fruit and two servings of vegetables per day and the avoidance of tea, coffee, red wine, fruit juices, nuts, seeds, vitamin or mineral supplements, and aspirin). There were 104 DEGs with an expression change of more than 1.5-fold. Antioxidant dietary treatment increased the expression of genes associated with the innate immune receptors (TLR2, IL1R2, CD93, and ANTXR2), innate immune signaling molecules (IRAK2, IRAK3, and MAP 3 K8), neutrophil proteases (MMP25 and CPD), and apoptosis regulation (CFLAR), while there was a reduction in the expression of FGFBP1, S100A16, IGFBP2, SLPI, CAPN9, MUC20, NDFIP2, DDR1, FOXA1, CLC, PIGR, CD24, FOXJ1, ALOX15, TFF3, TPSAB1, PROM1, GSTA1, GSTA2, C20orf114, and SERPINB3. Interestingly, sputum neutrophil counts were associated with the expression of TLR2, IRAK3, IL1R2, IRAK2, and CD93. Further, the expression of IRAK3, CD93, and IL1R2 significantly correlated to concentrations of antioxidants measured in plasma before the initiation of dietary treatment.

In a randomized, double-blind, placebo-controlled, crossover trial [19], 36 overweight men underwent an anti-inflammatory dietary treatment (resveratrol, green tea extract, α-tocopherol, vitamin C, n23 (omega-3) polyunsaturated fatty acids, and tomato extract) for 5 weeks. The inflammation-related metabolites, proteins, and genes significantly affected by the diet were classified into eicosanoid-related inflammation (omega-3/omega-6 eicosanoid precursor ratio, balance omega-3/omega-6 PUFAs, eicosapentaenoic acid, arachidonic acid, PTGIS, PGDS, HPGD, PTGFRN), inflammatory mediators and signaling molecules (adiponectin, prolactin, DPP4, TLR7, PTGIS, PGDS, HPGD, PTGFRN, SELP, IL10RA, SOCS3, inosine, uric acid, IL-18, CCL-22, MYOM1, IGHD, IGHV3-47, β2-microglobulin, IL12A), plaque formation and coagulation (factor VII, TNF RII, VEGFB, PEAR1, SELP, THBD, PF4, F10, PROCR, NAPG, FGF10, EDG2, PPBP), endothelial function (ICAM-1, VCAM-1, ANGPTL5, THBD, SMOC2), and blood cell differentiation (CSF-1, MPO, IL4R, CCL21, IL12A, IL8RB, IL7R, KLRG1, TESC, HBB, HBM, NKTR, ALAS2, IL15). There was a reduction in the omega-3/omega-6 eicosanoid precursor ratio as well as in plasma concentrations of arachidonic acid, prolactin, inosine, uric acid, IL-18, CCL-22, MYOM1, IL-12A, β2-microglobulin, factor VII, TNFRII, ICAM-1, VCAM-I, and CSF-1, whereas there was an increase in the balance omega-3/omega-6 PUFAs and in plasma levels of eicosapentaenoic acid, adiponectin, and MPO. In the adipose tissue, the expression of PTGIS, PGDS, HPGD, PTGFRN, DPP4, TLR7, PTGIS, PGDS, HPGD, PTGFRN, SELP, IL10RA, SOCS3, SELP, THBD, F10, PROCR, EDG2, ANGPTL5, THBD, SMOC2, and IL-15 increased, while the expression of PF4, NAPG, FGF10, PPBP, IL8RB, IL7R, KLRG1, TESC, HBB, HBM, NKTR, and ALAS2 decreased. In PBMCs, there was an increase in the expression of IGHD, VEGFB, and L4R as well as a reduction in the expression of MYOM1, IGHV3-47, IL12A, PEAR1, CCL21, and IL-12A.

The study [20] was comprised of a crossover trial and a parallel setup. In the former lean (n = 15, mean age: 36) and obese (n = 17, mean age: 40) subjects received low (50 g) and high (200 g) vegetable treatment in two consecutive periods of 4 weeks. All subjects, then, in the parallel received energy-restricted diet for 4 weeks. Following energy-restricted diet, neither the amount of weight loss nor the degree of reduction of plasma markers (total cholesterol, LDL cholesterol, ratio of cholesterol/HDL, c-GT, ASAT, and HbA1c) differed between lean and obese subject. Similarly, the superior efficacy of high vegetable consumption over low vegetable consumption in reducing fasting ASAT and ALP was observed among both lean and obese subjects, while only lean subjects revealed a decrease in the blood concentrations of TNFα. Analysis of the adipose tissue in different time points revealed changes in the expression of genes from low to high vegetable consumption. The number of DEGs affected by the amount of vegetable consumption was higher in lean than obese subjects (532 vs. 323). Particularly, DEGs in lean subjects included 40 genes related to inflammatory processes, immune responses, and chemo-

kine signaling pathway. Among them, there was an increase in the expression of SERPINA1, INS, LIG4, GBP6, CCR10, NCF1, CCL28, VNN1, IL8, LILRB5, NFKB2, TNFRSF13C, ADCY7, PSMB8, CD96, ITK, MR1, ALOX5, TBKBP1, HLA-DQA1, IL20RB, CNPY3, CD163, and F3, while the expression of CXCR5, NDST1, PLCB4, GNGT2, BCL3CASP6, VISA, TNFSF4, SP100, NFKBIB, MASP1, BTLA, LTF, CFH, CSF1, and C3 reduced. More interestingly was the difference between lean and obese subjects in weight loss-linked DEGs in response to energy-restricted diet. In lean subjects, DEGs were mainly correlated with inflammatory and immune responses, while they involved oxidative phosphorylation, energy metabolism, focal adhesion, and T-cell activation in obese subjects.

Comment

A single dose of curcumin and cranberry was enough to alter the expression of genes related to chemotaxis and inflammatory processes. Consistently, the gene expression response to repeated daily doses (2–5 weeks) of vegetables and fruits (hesperidin, orange juice, anti-inflammatory diet, anti-oxidative treatment, and high vegetable treatment) involved genes that contribute to the immune responses (especially innate immune responses), neutrophil proteases, chemokine signaling pathway, and inflammatory processes. As expected, both pave the way for anti-inflammatory response and thereby help the control of body weight and innate immunity-mediated diseases especially asthma.

Olive Oil

The study [21] investigated the effects of an acute consumption of 50 ml (44 g) of olive oil on the gene expression in PBMCs in six healthy men. Microarray analysis identified 259 and 246 genes that were differentially upregulated and downregulated by olive oil. Genes highly upregulated were involved in biological processes related to cancer (AKAP13 and IKAROS) and cellular processes (CDC14 and USP48), while genes highly downregulated were known to contribute to DNA damage (DDIT4 and XRCC4) and carcinogenesis (CDKN2B and AKT3). Of interest to the chapter were inflammation-related genes such as AKAP13, IL-10, SOS1, COL4A4, and STAT4 that were upregulated by olive oil consumption. There was, however, a reduction in the expression of genes, including CD69, IL-8, IFN-γ, CCL17, CXCR4, CLC, LEF1, and FPR1.

Whether the gene expression profile of PBMCs can distinguish the effect of high-polyphenols extra virgin olive oil (EVOO) from the effect of low-polyphenols EVOO was evaluated through a clinical study. There were patients with metabolic syndrome (n = 12) and healthy subjects (n = 12) who were administered a single dose (50 mL (44 g)) of EVOO. High-polyphenol EVOO induced a reduction in serum glucose, insulin, and HOMA-IR in healthy subjects, but not patients with metabolic syndrome. Such effect was not observed at all for low-polyphenol EVOO. The number of genes differentially expressed in response to high-polyphenol EVOO in healthy subjects was more than twice that in patients with metabolic syndrome (2447 vs. 954). Further interesting was that functional analyses indicated upregulation of genes involved in immune- and inflammation-related processes such as CD28 signaling in T helper cells and Fcγ receptor-mediated phagocytosis in macrophages and monocyte by high-polyphenol EVOO, while a downregulation in the expression of genes contributing to inflammation-related processes such as PI3K signaling in B lymphocytes, B-cell and T-cell receptors, NFAT, NF-κB, IL1, IL-3, IL-8, RANK, and thrombin signaling cascades, NRF2-mediated oxidative stress responses, and LPS-stimulated MAPK activation occurred as well. Supporting this, RT-PCR confirmed an increase in the expression of RXRβ associated with anti-inflammatory properties and a reduction in the expression of IRAK3 contributing to NF-κB and IL-8 signaling, CXCR4 related to autoimmunity, and HIF1A associated with inflammatory properties. None of these genes, except for HSPA1A, RXRβ, and CXCR4, were influenced by low-polyphenol EVOO. RT-PCT also showed an increase in the expression of miR-23b-3p as well as a reduction in the expression of miR-181b-5p which respectively exert anti-inflammatory and inflammatory effects [22].

A randomized controlled trial assigned 89 healthy subjects (age range: 20–50 years) to the following 3 dietary treatment groups: traditional Mediterranean diet (TMD) with virgin

olive oil (VOO), TMD with washed virgin olive oil (WOO), and control diet for 3 months [23]. WOO contains only 55 mg polyphenols per kg, while VOO has 328 mg/kg polyphenols. Overall, treatment with TMD led to the reduction in concentrations of isoprostanes in urine as well as in levels of glucose, HDL-C, IFN-γ, CRP, and s-P-selectin in blood. Subgroup analyses for the TMD plus VOO group not only confirmed these changes, except glucose reduction, but also showed decrease in the content of 8-oxo-dG in urine and in the amount of cholesterol in blood, whereas subjects who received TMD plus WOO only revealed reduction in CRP levels. Among inflammatory genes measured after 3-month dietary treatment, PBMCs from subjects who underwent TMD showed a decrease in the expression of IFN-γ as well as an increase in the expression of IL-7R compared to that of controls.

Comment

In addition to the direct effect of amount of polyphenols, studies indicate the significance of the metabolic status in determining the effects of olive oil on the expression of genes involved in immune and inflammatory responses. So that, the number of genes differentially expressed by olive oil decreased in patients with metabolic syndrome in comparison with metabolically healthy people.

Micronutrients

Another study on a sample ($n = 1007$) of the TNH study assessed the possible association between plasma concentrations of micronutrients and pro-inflammatory cytokines [24]. There were found significant associations between ascorbic acid and IL-1RA (r = 0.13), 25(OH) D and IP-10 (r = 0.12), 25(OH) D and PDGF-bb (r = 0.09), α-tocopherol and PDGF-bb (r = 0.08), 25(OH) D and RANTES (r = 0.22), ascorbic acid and RANTES (r = −0.07), and α-tocopherol and RANTES (r = −0.09), which all however were statistically weak.

A total of 22 subjects including 11 adults (mean age: 54.7) with adequate selenium (aSe) content and 11 adults (mean age: 53.6) with inadequate selenium (iaSe) content were included in the study [25] of a sample of Biomarkers of Risk of Colorectal Cancer (BORICC) data. When taking into account the cutoff of 1.2-fold change, expression profiling of rectal biopsies revealed a reduction in the expression of 126 genes as well as an increase in the expression of 128 genes in the group with iaSe compared to the group with aSe. Among the top downregulated genes, there were immune system-related genes including IL-1β, CCL2, CXCL8, CCL19. IPA identified immune pathways such as immune cell trafficking, inflammatory disease pathways, and immunologic disease pathways as affected by iaSe. Supporting this, analyses of the microarray/proteomics dataset put two immune-related networks among the most significantly affected networks: inflammatory response, cell death and survival, connective tissue disorders and antimicrobial response, inflammatory response, and cellular movement. Further interesting was that most of upstream regulators (PDGF BB, TNF, IL1B, SP1, TREM1, IL6, IFNG, NF-kB, TGFB1, CREB1, a-Catenin, STAT3, IRAK4, IgG, IL1A, PI3K, Jnk, P38, TLR3, Cg, TP53, ERK, CEBPA, RET, and FAS) predicted by IPA to be highly affected by iaSe were involved in the immune and inflammatory processes.

To study the effect of age on the gene expression in response to zinc, cells from 15 young and 16 elderly subjects underwent zinc treatment for 24 hours in the study [26]. Before zinc treatment, lymphocytes from elderly people demonstrated lower levels of zinc availability and NO-induced release of zinc compared to that from young subjects. However, zinc treatment led to an increase in the zinc availability in monocytes and lymphocytes as well as in the expression of metallothioneins in PBMCs from both young and elderly people. Further, NO-induced zinc release from lymphocytes and monocytes increased following zinc treatment in both young and elderly people. The expression of 68 and 61 genes was significantly altered by zinc treatment in young and elderly people, among which 14 were shared between both young and elderly subjects. In young subjects, 25 upregulated genes linked to lipid metabolism, molecular transport, and molecule biochemistry, whereas 23 downregulated

genes to cellular movement, cell-to-cell signaling and interaction, and tissue development as revealed by Ingenuity Pathway Analysis (IPA). In elderly people, 27 upregulated genes linked to cellular growth and proliferation, cancer, and cellular movement, whereas 23 downregulated genes to immune responses, cellular growth and proliferation, and tissue morphology.

Comment

Integrating relations between nutritional inadequacy and pro-inflammatory status from cross-sectional data as well as slowly shifting to anti-inflammatory status with nutrient supplementation would propose nutrients as a mechanism of preventing against inflammation.

Ethnic Dietary Patterns

Through a randomized controlled trial [27], healthy subjects eat either a tocopherol-enriched Mediterranean meal (TEM) or a Western high-fat meal (HFM). Higher oxLDL levels in plasma were associated with HFM as compared to TEM. The authors compared the expression of 13 genes: 6 related to the inflammasome pathway and 7 related to oxidative stress under baseline and TEM and HFM dietary conditions. Among the inflammasome pathway-related genes, the consumption of HFM was correlated with the increased expression of IRAK1 while the TEM with decreased expression of BCL2. When compared to the TEM, eating a HFM significantly reduced the expression of BCL2 and IRAK1.

The Toronto Nutrigenomics and Health (TNH) Study aimed at investigating the effect of ethnicity and respective dietary treatments on the content of proteins in plasma [28]. There were 1090 young adults (mean age: 22.7 years) from White, East Asian, South Asian, and other ethnical groups who consumed 1 of 3 main dietary patterns: Western, prudent, and Eastern. East Asians and South Asians showed a reduction in adiponectin levels compared to the Whites, while higher levels of apolipoprotein E, complement C1 inactivator, and histidine-rich glycoprotein were found in East Asians than Whites, and higher levels of complement C4 b and c chains and haptoglobin b were observed among South Asians than Whites and East Asians. Measured proteins ($n = 54$) were classified into four principal components, among which two were related to physiologic pathways (1 and 2), one to inflammation and innate immunity (3), and one to coagulation (4). Multiple regression analyses showed the positive association of principal component 3 with the Western dietary pattern in a crude model. The association became nonsignificant when several variables were included in the model. In the latter model, Western and Eastern dietary patterns showed positive and negative associations with principal component 1, respectively.

In a crossover design [29], 20 subjects 65 years old and older fed 4 isocaloric diets, low-fat, high-carbohydrate diet rich in n-3 PUFA (PO diet), Western diet rich in saturated fat (SFA diet), Mediterranean diet (MD), and Mediterranean diet supplemented with coenzyme Q (MDC), each one for a period of 4 weeks. Among genes related to the different elements of the ST/CORT system, the expression of sst3 in PBMCs was different between dietary treatment groups with higher sst3 levels in the SFA group. However, when post-prandial samples were analyzed, the expression levels of CORT, sst2, and sst5 were significantly higher in PBMCs from PO than that from MD, MDC, and SFA subjects.

Comment

Western high-fat diet correlates to the induction of immune genes and proteins involved in cardiovascular inflammation and apoptosis in comparison with the Mediterranean diet. Additionally, there are lower concentrations of proteins protective against atherosclerosis in White people who consume Western diet.

Calorie Restriction

As demonstrated by time series analyses (days 1, 2, 7, 14, and 28), a 28-day normocaloric diet decreased the blood expression of genes related to immunity and defense (B-cell- and antibody-mediated immunity, T-cell-mediated immunity, MHCI-mediated immunity, NK cell-mediated immunity, granulocyte-mediated immunity, cytokine/chemokine-mediated immunity, and interferon-mediated immunity) in three healthy men [30].

In [31], the gene expression was measured in PBMCs from obese men before and after an 8-week low-calorie diet (55% of energy as carbohydrates, 15% as proteins, and 30% as fat). There were 385 DEGs. Most of upregulated genes (n = 227) were known to contribute to nucleotide, DNA, and chromatin metabolism, cellular biosynthetic, and regulation of metabolic processes, while downregulated genes (n = 158) were mostly involved in signal transduction, cell communication, transport, immune response, and carbohydrate metabolism. Among inflammation-related genes, diet treatment significantly reduced the expression of TNIP1, TRIAD3, NKRF, RIPK3, and IL8, whereas the expression of TANK increased. Further interesting was that the expression level of these genes correlated to clinical parameters. Of note, there was an inverse correlation between the expression of IL-8 and fat mass decrease.

To address whether the pro-inflammatory status can predict weight loss regain, 84 obese subjects who underwent an 8-week low-calorie diet were followed for 6 months [32]. At the end of follow-up (week 32), almost half of patients (40/84) revealed weight lost regain >10%. When compared to those who maintained weight loss, subjects who regained >10% of the low-calorie diet-induced weight loss had higher circulating concentrations of TNFα as well as higher mRNA expression of NFKB1 and RELA in PBMCs at the end of dietary treatment (week 8). Logistic regression analysis revealed that elevated TNFα levels at week 8 were associated with more than threefold increased risk of weight loss regain at week 32. The risk even heightened when other potential variables (fat mass, age, and gender) were included in the model. The authors of the study then analyzed the expression of two specific inflammatory markers, RIPK3 and RNF216, in PBMCs from a sample of 12 of 84 obese subjects: six with weight lost regain >10% and six with weight lost regain ≤10% at week 32 [33]. The two groups did not differ in the expression of these markers at baseline (week 0). But the group with weight lost regain >10% had significantly higher mRNA expression of RIPK3 and RNF216 compared to the other group at the end of dietary intervention (week 8).

Comment

Both hypocaloric and normocaloric diets reveal to negatively regulate the expression of genes contributing to humoral and cellular immunity and inflammatory processes. This regulatory function aims at weight loss and its monitoring might aid in predicting weight loss maintenance.

Oral Challenge Tests

Study [34] of a sample (n = 8) of the MECHE study compared the gene expression profiles in PBMCs and white adipose tissues upon oral lipid tolerance test (OLTT) and oral glucose tolerance test (OGTT) on separate days. These two profiles were significantly comparable to each other. More interesting the top ranked DEGs of PBMCs and adipose tissues overlapped in genes related to the inflammatory (TLR4, NLRP3, and CASP1) and metabolic (PPARA, PPARG, RXRA, and NR1H3) processes. The 2D plot of PBMCs drawn by CIA (co-inertia analysis) included X-axis genes such as IL-8, FOS, and FOSB associated with nutrient responses and Y-axis genes consisted of CCL2, IL-1β, and EGF correlated with immune and inflammatory responses. In the plot of adipose tissue, the X-axis was composed of genes CD69, IL1B, and FFAR2 related to immune response and leukocyte activation and Y-axis genes consisted of SAA1, PDGFD, and IL18 involved in acute phase response and cell–cell signaling.

The Metabolic Challenge (MECHE) study in Ireland investigated the effect of oral lipid tolerance test (OLTT) on gene expression in PBMC of healthy male and female individuals (mean age: 33 years) [35]. A total of five genes showed altered expression, among which three were shared between males and females: PER1, DDIT4 and FKBP5. The other two genes DHRS9 and EREG were differentially altered in females. As gene Ontology (GO) functional analysis indicated, several gene co-expression networks are involved in response to OLTT, among which energy-related module and inflammation and immune-related module were specifically associated with OLTT in females. Favorable analyses highlighted the NLR Family Pyrin Domain

Containing 3 (NLRP3) as a top hub gene in the inflammation- and immune-related module. Further interesting was the female-specific association of inflammatory-related module with lower levels of cytokines IL-2 and IL-10 as well as with higher levels of glucose in plasma.

When human umbilical vein endothelial cells (HUVECs) were cultured with serum from healthy subjects ($n = 7$) after an oral fat challenge with fresh cream [36], there were 675 DEGs; among them 36 (LY6D, CUBN, LRMP, MMD2, SERPINC1, CXCR3, PTGER2, PGLYRP1, SLC44A1, IGSF9, IFITM3, VPREB1, SERPINA10, F2RL2, PCDHB13, PTGFR, IL4, CD163, CDC6, TKTL1, PGF, IFIT2, CDC34, CCL3L3, CDH23, LILRA2, IL3, TNFAIP8L2, CDC42EP5, CRB1, CDC26, KLRD1, NGFR, ITGB1BP2, FGA, and SCARF2) were classified in the category of immune- and inflammation-related processes. The challenge also induced an increase in plasma concentrations of TG and FFA.

Comment

Evidence is conclusive about the impact of oral fat and glucose administration on the expression of inflammatory genes.

Hormone Therapy

In a female sample ($n = 783$) of the TNH study, the effect of hormonal contraceptive (HC) on the protein content in plasma was investigated [37]. 19 of 55 measured proteins were significantly different between HC users ($n = 240$) and non-users ($n = 543$). As compared to non-users, HC users showed an increase in the levels of α1-Antitrypsin, Angiotensinogen, α2-HS-Glycoprotein, apolipoprotein A-I, apolipoprotein A-II Precursor, apolipoprotein L1, CRP, ceruloplasmin, Vitamin D Binding Protein, coagulation Factor XIIa HC, heparin cofactor II, Kininogen-1, plasminogen, Retinol-Binding Protein, serum Amyloid P-Component, and Vitronectin, while there was a reduction in the levels of apolipoprotein E, Complement C1 Inactivator, and Histidine-rich Glycoprotein HC users than non-users. Further interesting was that the protein content was influenced by dose and duration of HC. However, this influence became nonsignificant after Bonferroni correction.

Comment

Hormone therapy causes aberrations in proteins contributing to the link between inflammation and cardiovascular disorders and so poses a threat to cardiovascular health.

Animal Studies

Vegetables, Fruits, and Other Plant-Derived Products

A feeding trial categorized 280-day-old Ven Cobb broilers into seven dietary groups for 6 weeks: basal diet (T1), basal diet supplemented with 0.5% garlic powder (GP) (T2), basal diet supplemented with 1% GP (T3), basal diet supplemented with 0.5% holy basil leaf powder (HBLP) (T4), basal diet supplemented with 1% HBLP (T5), basal diet supplemented with 0.5% GP and 0.5% HBLP (T6), and basal diet supplemented with 1% GP and 1% HBLP (T7) [38]. When compared to the other dietary groups, T6 and T7 diets brought boilers best weight gain and feed conversion ratio. Boilers who received the T5 diet showed highest expression of TLR-2 and TLR-4 in plasma, while the expression of TLR-7 was lowest in these groups as compared to other six dietary treatment groups. Instead, the highest expression of TLR-7 was observed in boilers which consumed the T3 diet.

The gene expression response to treatment with different nutraceuticals (EA, *Echinacea angustifolia*; VM, *Vaccinium myrtillus*; CL, *Curcuma longa*; SM, *Sylibum marianum*) for 60 days was investigated in [39]. To this end, there were one control dietary group and four other dietary groups of dogs for each of the mentioned nutraceuticals. At T60, treatment with VM, CL and EA, but not SM, significantly reduced the expression of TNF-α and NFKB1 in white blood cells as compared to the control group. Also, VM and CL groups showed lower expression levels for CXCL8 and PTGS2 than the control group, whereas there was an increase in the expression of CXCL8 in the EA group compared to the control group. All the dietary groups except for those eat EA revealed higher expression of SOD1 than the control group.

Studies in apolipoprotein E-deficient mice aimed to investigate possible mechanisms of action of the anti-atherosclerotic effect of Bilberry anthocyanin-rich extract (BE) [40, 41]. Mice were allocated to the control group or the intervention group which fed the control diet plus 0.02% BE for 2 weeks. Inclusion of BE in the diet decreased plasma levels of cholesterol and hepatic content of TG. Treatment with BE was associated with altered expression of 2289 genes. Pathway analyses identified immune signaling pathways and inflammation-related processes as possible mechanisms of protective action of BE. The inflammatory genes were related to B- and T-cell receptor signaling pathways, TLR signaling pathway, NK cell-mediated cytotoxicity, and complement and coagulation cascades. Also, the genes related to signaling pathways such as Jak-STAT signaling pathway, cytokine-cytokine receptor interaction, and MAPK signaling pathway were involved in immune responses. Finally, the top anti-atherosclerotic effect of BE-associated genes was four of inflammation-related genes: ALOX5AP, CX3CL1, TNFRSF14, and C3.

In the attempt to evaluate the effects of phytonutrients on the gene expression profile, 1-day-old chickens were allocated to one of the four following dietary treatments: control diet alone, control diet supplemented with carvacrol (5.0 mg/kg), control diet supplemented with cinnamaldehyde (3.0 mg/kg), and control diet supplemented with Capsicum oleoresin (2.0 mg/kg), for a week [42]. Five genes (CD74, CDC5L, UBE2I, FADD, and CDK5RAP2) were selected among DEGs identified by microarray analyses of intestinal intraepithelial lymphocytes. RT-PCR confirmed the increase in the expression of CD74 and CDK5RAP2 as well as reduction in the expression of CDC5L, UBE2I, and FADD by Capsicum oleoresin. Also a network of the best associated genes was defined for cinnamaldehyde. It mainly included the genes that are involved in antigen presentation, humoral immune response, and inflammatory disease.

Healthy and varroa-parasitized local hybrid honeybees fed either a control diet without pollen or control diet with pollen [43]. At the transcription level, when compared to the healthy bees, Varroa parasitism mostly reduced the expression of genes consistent with decreased metabolism and weight. Among four candidate genes for RT-PCT, prophenoloxidase (PPO) and spaetzle were related to immunity. Pollen feeding increased the expression of PPO and spaetzle in healthy bees. However, it was not statistically significant for PPO. In varroa-parasitized bees, pollen feeding had no significant effect on the expression of these genes. Among bees which received the control diet without pollen, those who were varroa-parasitized had lower expression of PPO, but not spaetzle, than their healthy counterparts, while, among bees which received the control diet with pollen, parasitized bees demonstrated reduction in the expression of both PPO and spaetzle. Pollen feeding upregulated the expression of immune processes-related genes such as lysosyme-2 and lysosyme-3, PSH, SPZ, PGRP-LC, and Defensin-1 in healthy bees, while in parasitized bees, the only gene whose expression was upregulated by pollen feeding was lmd. Among bees which received the control diet without pollen, there was an increase in the expression of NEC, cact-1, Defensin-1, PSH, Hymenoptaecin, Apidaecin 1, and Lysosyme-3 in varroa-parasitized than healthy bees, while, among bees which received the control diet with pollen, parasitized bees demonstrated upregulation in the expression of Defensin-1, PSH, and Hymenoptaecin compared to non-parasitized bees. Pollen feeding downregulated the expression of several immune genes including Hymenoptaecin, Imd, Domeless, GNBP-1, PGRP-SC2, Pellino, cact-3, PSH, Kay, Tab, Hopscotch, and TEPs in healthy bees. In parasitized bees, the genes whose expression was downregulated by pollen feeding were Hymenoptaecin, Lysosyme-3, Domeless, GNBP-1, PGRP-SC2, Pellino, cact-3, PSH, NEC, Myd88, PPOact, Stat92E, and Apidaecin 1. Among bees which received the control diet without pollen, there was a decrease in the expression of PPO, lap2, Ird5, PGRP-SC2, Pellino, Tab, Kay, Myd88, Lysosyme-1, cact-3, PPOact, GNBP-1, Domeless, and Hopscotch in varroa-parasitized than healthy bees, while, among bees which received the control diet with pollen,

parasitized bees demonstrated reduction in the expression of PPO, lap2, Myd88, Lysosyme-1, cact-3, PPOact, GNBP-1, Domeless, Apidaecin 1, Lysosyme-3, PSH, SPZ, NEC, Toll, NEC, PGRP-LC, Lysosyme-2, dorsal-1, Stat92E, TEPs, and Hopscotch compared to non-parasitized bees.

Comment

The tuning effect of plants on the expression of genes involved in the immune responses and signaling pathways centers with important implications for anti-atherosclerotic bioactivity, antiviral defense, and anti-autoimmune effects.

Fish Oils and Meals and Plant Oils and Meals

To evaluate the effect of flaxseed oil on cardiovascular and inflammatory biomarkers, the study [44] included mice in three different dietary patterns, control diet (CD), high-fat (HF) diet, and HF diet plus flaxseed (FS) oil, scheduled for 8 weeks. Generally, HF diet demonstrated to be deleterious to the different metabolic parameters, e.g., food intake, body weight, glucose tolerance, insulin sensitivity, LDLc, and total cholesterol. Inclusion of FS oil into the HF diet prevented the increase in food intake, body weight, and LDLc as well as resulted in higher HDLc. Analysis of aorta from the HF diet plus FS oil revealed reduction in expression of inflammatory (IL-1β, TNF-α, and IKK) and endoplasmic reticulum (ER) stress-related (ATF-6 and GRP78) genes in comparison with that of HF diet. However, in the LDLr-KO mouse, FS was not able to abolish the negative effects of HF diet on metabolic parameters and the expression of inflammation- and ER stress-related genes as much as did in the wild-type Swiss model.

Atlantic salmon underwent 7.4% (FO7) or 5.1% (FO5) fish oil for 16 weeks [45]. Generally, the head kidney leukocytes (HKL) from salmon on FO5 diet had higher proportion of free fatty acids and sterols and lower proportion of phospholipids. Particularly, there was an increase in the sum of long-chain n-6 fatty acids (LCn-6) and the LCn-6/LCn-3 ratio as well as in the contents of linoleic (18:2n - 6), α-linolenic (18:3n - 3), and dihomo-gamma-linolenic acids (20:3n-6) in the HKL from FO5 diet, while there was a reduction in the content of tetradecanoic (14:0), pentadecanoic (15:0), eicosenoic (20:1n-9), and eicosapentaenoic (20:5n-3) acids. Microarray analyses of salmon MLCs identified a series of particle-in-cell (pIC)-responsive transcripts that were similarly associated with both FO7 and FO5 diets, among which immune responses-associated transcripts involving chemokine receptor activity, cytokine receptor activity, cellular response to cytokine stimulus, chemokine-mediated signaling pathway, response to cytokine, negative regulation of erythrocyte differentiation, megakaryocyte differentiation, TRIF-dependent toll-like receptor signaling pathway, MyD88-independent toll-like receptor signaling pathway, inflammatory response, adaptive immune response, and cytokine-mediated signaling pathway were overpresented, while toll-like receptor 3 signaling pathway and negative regulation of type I interferon production were only among pIC-responsive transcripts in the group of FO7 diet, but not FOD diet. Meanwhile, the MLCs of salmon in the group of FO7 diet showed increase in the expression of proteasome subunit beta type-8 (psmb8) and reduction in the expression of fatty acid-binding protein adipocyte (fabp4) as compared to that in the group of FO5 diet.

The Atlantic cod were allocated to one of the three experimental dietary groups: control diet (HO: herring oil), 40% (CO40), and 80% (CO80) replacement of HO for 67 days [46]. Dietary treatment affected neither growth parameters nor splenic gene expression profiles (baseline or in response to immune stimulation by pIC). Of note was the higher expression of METTL6 in spleen from CO40 compared to that from CO80 or HO. It was the only gene whose expression was significantly different between dietary groups. Within-group GO analysis of pIC-responsive DEGs revealed the overpresentation of genes associated with immune system process, regulation of immune response, innate immune response, pattern recognition receptor signaling

pathway, cytokine production, defense response to virus, antigen processing and presentation, RIG-I signaling pathway, and toll-like receptor signaling pathway in all the three diet groups.

Rainbow trout consumed either a fishmeal- or a plant meal-based feed for 12 weeks [47]. Immune responses-related genes were one of the main categories of genes that were differentially expressed.

Comment

The plant oils produce effects opposite to inflammation and cellular stress, while fish oils have demonstrated to induce the expression of genes involved in the inflammation and innate and adaptive immune responses (cytokine production, anti-viral defense, antigen processing and presentation, chemokine and cytokine receptor activity, cellular response to cytokine stimulus, response to cytokine, negative regulation of erythrocyte differentiation, and megakaryocyte differentiation) and related signaling pathways (pattern recognition receptor signaling pathway, chemokine-mediated signaling pathway, TRIF-dependent TLR signaling pathway, MyD88-independent TLR signaling pathway, cytokine-mediated signaling pathway, and RIG-I signaling pathway).

Protein Sources

Atlantic salmon consumed one of the following ten dietary treatments: corn gluten 2 g/kg ± soyasaponin supplementation, pea protein concentrate ± soyasaponin supplementation, sunflower meal ± soyasaponin supplementation, rapeseed meal ± soyasaponin supplementation, and horsebean meal ± soyasaponin supplementation for 80 days [48]. As compared to the group fed with Pea protein concentrate alone, inclusion of soyasaponin in Pea protein concentrate led to the development of distal intestinal changes in mucosal fold fusion (bridging), connective tissue hyperplasia, leukocyte infiltration in the lamina propria and submucosa, supranuclear absorptive vacuolization and abnormal nucleus position in enterocytes, and numbers of goblet cells, while there were only subtle histological changes in mucosal folds and lamina propria for the fish in the group of rapeseed meal and in the numbers of goblet cells for those in the group of sunflower meal. In fact, the intestinal histology was most influenced by Pea protein concentrate supplemented with soyasaponin as compared to other dietary treatments. At the level of transcription, DEGs in response to Pea protein concentrate supplemented with soyasaponin involved in the various biological processes mainly inflammation (chemokines and complements), metabolic pathways (amino acid, steroids, and lipids), cellular and tissue structures (cell surface, lysosome, mitochondrion, peroxisome, and basal membrane), and integrative functions (hormone activity and digestion). Of interest to the present chapter are DEGs involved in immune responses and inflammatory processes. They could be categorized into inflammatory mediators and transducers (IL-22; IL-18; CK-1; CCL19; SOCS; CCR9; CCL21; IL-6R1; IL-1R2; Il-1RA; ALOX5AP; LTB4 12-HD; Annexins A1, A2-A, and A5; TNFRs; TNRS5; TNFAIP8L2; NFKB p100 subunit; NFKBIA; NFKBIE; C/EBPβ; AP-1; and jun-B), IFN-dependent (MHC class I antigen, MHC class I, MHC class Ia heavy chain, B2M, Jak1, similar to very large inducible GTPase 1, RTP3, GILT, IFI44, FinTRIM, SRK2, and LGALS3BP), effectors: complement and lectins, antimicrobial proteins (FBPL4, Precerebellin-like protein, CFD, C1QL2, C1QL4, C5AR1, C6, PFC, C1-inh, C4BP, Nattectin, Cathelicidin, FCER1G, Fcgr1, DRTP1, and Clr-a), proteases and inhibitors, T-cells (LINCR, MMP9, Collagenase 3, MMP, ELF3, TIMP2, SERPINB1, V-TCR, TIMD4, CD86, and CTLA4-like protein), and oxidative burst, protection against free radicals (MPO, CYBA, CYBB, NCF1, ARG2, ODC1, NOSTRIN, GSR, GPX4, GSTA3, GSTK1, GSTP1, GSTZ1, PRDX4, Catalase, ARRDC2, CISD1, MSRA, ALAS1, MTA, HO, HEBP2, Ferritin middle subunit, SLC31A1, and ATOX1). All the genes, but CCL21, C1-inh, and C4BP, related to inflammatory mediators and transducers, effectors, and proteases and inhibitors were differentially upregulated in response to Pea pro-

tein concentrate supplemented with soyasaponin. By contrast, all the genes, but Jak1, involved in the IFN axis were downregulated. Among genes related to oxidative burst and protection against free radicals, the expression of MPO, CYBA, CYBB, NCF1, ARG2, ODC1, GSR, and ALAS1 increased and the rest of them were reduced.

In an 87-day feeding trial [49], Atlantic salmon received one of four dietary treatments that contain progressively increasing proportions of solvent-extracted soybean meal (SBM: 0 g/kg, 100 g/kg, 200 g/kg, and 300 g/kg). Fish that fed the diet with 200 g/kg SBM showed growth retardation compared to those that fed 0 or 100 g/kg SBM and fish that fed the diet with 300 g/kg SBM had the worst growth performance. Gene transcription analyses indicated that intestinal tissue generates a noticeably better-defined response to SBM than the liver tissue as reflected in higher numbers of DEGs and higher degrees of expression change. There were more than 1900 transcripts in the intestine vs. 133 transcripts in the liver whose expression changed more than 1.3-fold. Among those with the highest changes in the liver were genes involved in the complement and coagulation cascades. Particularly there was an increase for the expression of C3 and C7 and to a lesser extent for C5 and C6. As mentioned, SBM affected the intestinal expression of genes in many ways. Among the most significantly DEGs were immune system-related genes. Precisely, SBM upregulated the expression of genes contributing to the TNFα signaling pathway NOD-like receptor interaction, NF-kB signaling pathway, cytosolic DNA sensing pathway, Jak-STAT signaling pathway, cytokine-cytokine receptor interaction, and T-cell receptor signaling pathway.

Atlantic salmon were assigned to the control diet alone (negative control) or to one of the dietary treatments that contained SBM (positive control diet), 0 g/kg, 111.8 g/kg, 223.6 g/kg, 335.4 g/kg, or 447.2 g/kg protein concentrates from faba bean (BPC) for 8 weeks [50]. Inclusion of 223.6 g/kg or higher BPC had a negative effect on growth performance. Histological analysis of posterior intestine indicated that inclusion of SBM and 447.2 g/kg BPC raised the scores of supernuclear vacuoles (SNV) and goblet cells (GC) in enteritis assessment. When lower amounts of BPC were included, the intestinal histology was comparable to that of the negative control diet. Similarly, transcription analysis in the liver tissue showed a pronounced response to diets consisting of SBM or 447.2 g/kg BPC but not of lower amounts of BPC. More interesting was the more the amount of BPC is included, the greater the number of DEGs. Of note was the overpresentation of genes related to complement cascades (C4, C5, CR3b/4b, C1, C8) among genes whose expression differentially altered in response to BPC not SBM.

In the study [51], rats were allocated to dietary treatments which contain different kinds of proteins including casein, soy, pork, fish, or chicken. There were 308, 53, 10, and 9 proteins differentially expressed in response to chicken, soy, fish, and pork proteins as compared to casein protein. Both chicken and soy protein diets resulted in a reduction in the expression of proteins associated with fatty acid metabolism. The chicken protein diet increased the expression of proteins involved in glucose and branched chain AA metabolism, while the soy protein diet induced the expression of proteins related to alanine and aspartate metabolism. At the level of protein sets, there were 41, 36, 28, and 22 protein sets differentially expressed in response to soy, fish, pork, and chicken proteins as compared to casein protein. The only dietary protein that negatively regulated the expression of immune system-related proteins was the chicken protein.

Comment

The soybean is the best investigated among different protein sources. Dietary supplementation with the soybean has demonstrated to upregulate the expression of genes contributing to inflammatory responses.

Fatty Diets

In the attempt to investigate the possible molecular mechanisms of obesity, mice consumed con-

trol diet or high-fat diet for 0, 2, 4, 6, 8, 12, 20, and 24 weeks [52]. The hepatic gene expression at the mentioned points of time was measured, and accordingly DEGs in response to high-fat diet could be categorized into patterns 1–8: (1) long-term upregulated (cellular assembly and organization and immunological disease), (2) long-term downregulated (lipid metabolism), (3) early upregulated (gene expression and inflammatory response), (4) early downregulated (cell signaling), (5) late upregulated (lipid metabolism, molecular transport, and small molecule biochemistry), (6) late downregulated (protein synthesis and cell-to-cell signaling and interaction), (7) early upregulated and late downregulated (cell-to-cell signaling, cellular growth and proliferation), and (8) early downregulated and late upregulated.

In the study [53] mice received control diet, 1-week high-fat diet (T1), and 2-week high-fat diet (T2). Microarray studies of cecum samples identified only seven DEGs in response to high-fat diet at T2. Among them, three were related to inflammatory processes (Fst, Tspan4, and H2-Q10) and four (Bmal1, Nr1d2, Tef, and Hlf) linked to the circadian clock. Compared to the control diet, 2-week high-fat diet relatively decreased the expression of Fst, H2-Q10, and Bmal1 whereas increased the expression of Tspan4, Nr1d2, Tef, and Hlf.

The effect of Western diet (10–14 weeks) on atherosclerosis was investigated in 12/15-LOX knockout mice, apoE knockout mice, and 12/15-LOX and apoE double-knockout mice [54]. After 10-week Western diet feeding, the size of atherosclerotic lesions in the double-knockout mice was diminished as compared to that in the apoE knockout mice. Strikingly, the effect became nonsignificant after 14 weeks. Also, at 15 weeks after transplantation, female mice which received BMT from the double-knockout mice developed smaller (however not significant) lesions than those which received BMT from the apoE knockout mice. When compared to that treated with plasma from apoE knockout mice, there was a fivefold increase in the TNFα-induced ICAM-1 in HUVECs treated with plasma from the 12/15-LOX and apoE double-knockout mice which fed low-fat-chow-fed. But such difference between groups of mice was not found for the Western diet. In comparison to the wild-type mice, the 12/15-LOX knockout mice showed increase in the expression of CCL5, CD18, and TGF-β as well as a reduction in the expression of IL-12p40 in macrophage. Experiments demonstrate that decreased production of LXA_4 is at least partly responsible for such changes. Further, after Western diet feeding, the plasma concentrations of pro-inflammatory cytokines such as IFN-γ, IL-2, and IL-17 increased in the 12/15-LOX knockout mice compared to the wild-type mice.

Two-month aged rats were treated with one of the following dietary treatment groups: (a) control diet for 4 months, (b) hyperlipidic diet for 4 months, (c) cafeteria diet plus control diet for 4 months, and (d) cafeteria diet plus control diet for 2 months and then control diet alone for another 2 months [55]. In time, feeding cafeteria diet plus control diet demonstrated to progressively increase body weight, adiposity, and serum leptin levels while decreasing TG levels. Similarly, these effects were observed for animals which fed HF diet. The exception was increased body weight which began to normalize at the second month of diet. Animals which received cafeteria diet revealed higher expression of TNFα at 4–6 months of age, while this effect was observed only at 4 months of age in rats treated with the HF diet.

Comment

The bad nature of hyperlipidemic diet is well-represented with early aggravation of genes related to inflammatory responses and is followed by induction of genes associated with immune-mediated diseases. The profile of cytokine gene expression has the potential to reflect the effect of high versus low-fat diet on the immune system.

Micronutrients

To assess the gene expression response to selenium supplements, ten lactating crossbred ewes in [56] first lived on a basal diet for 4 weeks

(T0) and then continued the basal diet supplemented with organic selenium for 40 days (T40). Microarray analysis identified significant difference in the expression of 942 and 244 transcripts between T40 and T0. Favorably, functional analysis asserted enrichment of differentially expressed genes that are known to contribute to different immune functions (lymphocyte activation, cytokine binding, leukocyte activation, T-cell differentiation, B-cell activation) and signaling pathways (cytokines and B- and T-cell receptor signaling pathways).

Rats received one of the four following diets for 12 weeks: CuA (125 mg/kg Cu)/10% sucrose (control diet), CuD/30%- Cu deficient (<0.3 mg Cu/kg)/30% sucrose, CuA/30%- Cu adequate (125 mg/kg)/30% sucrose, and CuD/10%- <0.3 mg/kg Cu/10% sucrose) [57]. The hepatic expression of transcripts following dietary treatment was compared with that of control diet. In the category of immune and inflammation-related processes, genes differentially expressed in response to CuA/30% were related to the TNF family (Tnf, Ltb, Tnfsf13b, Tnfsf15, Tnfrsf8, Tnfrsf9, and Tnfrsf21), T helper cell cytokines/receptors (IL-4, IL-2ra, IL-17rd, IL-21r, and IL-22ra1), chemokines (Ccl2, ccl3, ccl7, cxcl1, cxcl2, cxcl13, and cxcr2), extravasation and infiltration (Cldn11, Cldn20, sele, sell, and selp), extracellular matrix remodeling (Col6a3, Col11a2, Hs6st2, Hs6st3, Lama1, Mmp17, Mmp25, Sult4a1, timp4), and other (Csf1r, Csf2rb, Csf3r, Il-1r2, Il-7r, Il-12rb2, Lbp, and osmr). The expression of all of them increased, except cxcl13 whose expression decreased. CuD/10% induced the expression of genes involved in TNF family (Tnfsf13b, Tnfsf15, Tnfrsf9, and Tnfrsf21), T helper cell cytokines/receptors (IL-4, IL-2ra, IL-21r, and IL-22ra1), chemokines (cxcr2), extravasation and infiltration (Cldn9, Cldn17, Cldn19, Cldn20, Itga2, and sele), extracellular matrix remodeling (Col6a3, Hs6st2, Hs6st3, Lama1, Mmp25, Sult4a1, timp4), and other (Csf1r, Csf2rb, Csf3r, Il-12rb2, Lbp, osm, and osmr), while CuD/30% only resulted in the upregulation of Tnfsf15, Il-2ra, Sele, Lama1, and Csf3r. In addition to the inflammatory pathways, DEGs were associated with fibroblast proliferation and differentiation, markers of HSC activation, and metabolism and Mets-related. Altogether, copper deficiency and sucrose challenge switched the hepatic expression of genes in favor of inflammation and fibrogenesis, so that NAFLD develops.

Comment

Nutrient (selenium and copper) supplementation has shown to increase the expression of genes, thus enhancing the host immunity, while nutrient deficiency eventuates in the expression of genes linking inflammation and pathologies such as liver diseases.

Nutritional Stress

In a crossover trial [58] on influence of dietary constraints on the expression of eight selected genes including two (TGF-β and IL-1) related to immune response, four female Steller sea lions underwent an almost 70-day trial composed of three consecutive diets: normal food intake (NFI) for at least a month, a 35% decrease in NFI for 14 days (acute nutritional stress), and maintenance food intake for 28 days (chronic nutritional stress). PBMCs reflected a significant increase in the expression of IL-1 early after acute nutritional stress. However, reduction in the expression of TGF-β did not appear until the end of chronic nutritional stress.

In the study [59] cows were assigned to either the overfeeding group or the control group during the 45-day dry period. The former received over 140% calculated NEL (OVE; 1.62 Mcal/kg DM) from a corn silage–based diet and the latter received "at least 100% calculated NEL (CON; 1.34 Mcal/kg DM) from a diet high in wheat straw." Microarray analysis of the PMN identified more than 1800 differentially expressed genes (DEG), among which there were 139 genes with more than threefold change relative to control. Interestingly, TLR5 was the second top-upregulated genes in the overfeeding group. Accordingly, pathway analysis highlighted top pathways involved in response to overfeeding. The pathways including glycosaminoglycan biosynthesis – chondroitin sulfate and amino-

acyl – tRNA biosynthesis, one carbon pool by folate, RNA degradation, thiamine metabolism, ribosome, biosynthesis of unsaturated fatty acids, oxidative phosphorylation, and spliceosome were relatively more activated in the PMN from cows in the overfeeding group, while the relatively deactivated pathways were ubiquinone and other terpenoid-quinone biosynthesis, RNA polymerase, circadian rhythm, RNA transport, proteasome, ribosome biogenesis in eukaryotes, toll-like receptor signaling pathway, pyrimidine metabolism, osteoclast differentiation, and mineral absorption.

Comment

Altered cytokine and TLR signaling reflect the effect of nutritional stress.

Fatty Acid and Other Supplements

The effect of diet was investigated in the IL-10 knockout mouse model of inflammatory bowel disease. Mice were assigned to one of the dietary treatment groups: AIN-76A (5% corn oil), AIN-76A (fat-free) + 1% corn oil +3.7% oleic acid (OA), AIN-76A (fat-free) + 1% corn oil +3.7% AA, and AIN-76A (fat-free) + 1% corn oil +3.7% EPA [60]. As compared to the control diet AIN-76A, both AA- and EPA-enriched diets led to the expression of genes (DEGs) that are involved in cell-to-cell signaling and interaction, inflammatory diseases, and amino acid metabolism. Meanwhile the OA diet was associated with activation of similar pathways including cellular growth and proliferation, cell-to-cell signaling and interaction, and immune response. When the EPA-enriched diet was compared to the control diet, the colonic expression of inflammation-related genes such as ABCB4, CAST, MGLL, MYLK, PPARα, and SLPI increased, whereas the expression of TNFRSF1B decreased. In comparison to the control diet, there was an increase in the expression of ABCB4, CAST, CD38, ICOSLG, CAST, MGLL, MYLK, PPARα, PPARGC1A, and PRSS23 as well as a reduction in the expression of FGF7, IL-6, PTGS2, S100A8, and TNFα in the AA-enriched diet group of mice.

In the trial [61], 80-day-old broiler chicks received control diet alone or supplemented with insulin for 34 days. Taking into account only those genes, which were then included in the functional annotation analyses, the hepatic gene expression profile in birds which fed control diet supplemented with insulin demonstrated an $\geq$1.4-fold increase in the expression of 95 genes as well as a $\leq$ 0.6 reduction in the expression of 35 genes compared to the birds which fed control diet without insulin supplementation. Among the upregulated genes, there were genes related to immune processes. RT-PCR provided evidence to verify the upregulation of four (ITIH5, DIO2, KIAA1754, and GIMAP5) of six selected genes.

In the nutrigenomic study [62], rats received control diet, 15% fructose treatment, or 15% fructose plus an omega-3 fatty acid diet rich in DHA for 6 weeks. Animals treated with fructose revealed elevated blood glucose, triglycerides, insulin, and insulin resistance index that their counterparts on the control diet. More importantly, they demonstrated impaired memory performance. By contrast, when compared to controls, animals treated with 15% fructose plus an omega-3 fatty acid diet rich in DHA showed a decrease in blood triglycerides, insulin, and insulin resistance index and improvement in memory performance. Supporting this, functional analyses of hippocampal transcripts revealed genes that express differently in response to fructose compared with DHA.

Comment

Fatty acids differentially affect the expression of genes related to inflammation responses as well as lipid and glucose profile. Hence, their selective inclusion/ exclusion can be adopted as dietary treatment for IBD.

Conclusions

The first series of SNP studies establish the interaction between polymorphisms within the genes related to immune and inflammatory responses (CRP, IL-1, TNFα, IL-6, LTA4, and SCD-1) and diet composition (vitamin D status,

botanical formulation, fat intake, and fatty acid supplementation). Consequent changes in the content of inflammatory markers (CRP, IL-6, TNFα, and IL-1β), lipids (triglycerides), and fatty acids (palmitic acid, oleic acid, palmitoleic acid) may contribute to the pathophysiology of vascular and metabolic disorders. The second series of SNP studies show that the interaction between polymorphisms within the genes related to metabolic pathways (GCKR, Fok-1, and FADS 1/2) and diet composition (fat intake, nutritional counseling, and vitamin D therapy) would influence the content of inflammatory markers (IL-6, CRP, and α2-microglobulin), fatty acids (dihomo-γ-linolenic acid and arachidonic acid), and lipid (HDL, LDL, and oxLDL) and sugar profile (fasting glucose and HbA1c). In this manner, these interactions might confer susceptibility to the metabolic disorders particularly diabetes and NAFLD. Human and animal studies demonstrate that the tuning effect of plants on the expression of genes involved in the immune responses, inflammatory processes, and related signaling pathways centers with important implications for anti-atherosclerotic bioactivity, anti-viral defense, and anti-autoimmune effects. It might help the control of body weight and innate immunity-mediated diseases especially asthma as well. In addition to the direct effect of amount of polyphenols, studies indicate the significance of the metabolic status in determining the effects of olive oil on the expression of genes involved in immune and inflammatory responses. Generally, the plant oils produce effects opposite to inflammation and cellular stress, while fish oils have demonstrated to induce the expression of genes involved in the inflammation and innate and adaptive immune responses and related signaling pathways. Integrating relations between nutritional inadequacy and pro-inflammatory status from cross-sectional data as well as slowly shifting to anti-inflammatory status with nutrient supplementation (zinc, selenium, and copper) would propose nutrients as a mechanism of preventing against inflammation and pathologies such as liver diseases. Western high-fat diet correlates to the induction of immune genes and proteins involved in cardiovascular inflammation and apoptosis in comparison with the Mediterranean diet. Additionally, there are lower concentrations of proteins protective against atherosclerosis in White people who consume Western diet. The bad nature of hyperlipidemic diet is well-represented with early aggravation of genes related to inflammatory responses and is followed by induction of genes associated with immune-mediated diseases. The profile of cytokine gene expression has the potential to reflect the effect of high versus low-fat diet on the immune system. Both hypocaloric and normocaloric diets reveal to negatively regulate the expression of genes contributing to humoral and cellular immunity and inflammatory processes. This regulatory function aims at weight loss and its monitoring might aid in predicting weight loss maintenance. Altered cytokine and TLR signaling reflect the effect of nutritional stress (nutritional constraints and overfeeding). Hormone therapy causes aberrations in proteins contributing to the link between inflammation and cardiovascular disorders and so poses a threat to cardiovascular health. Dietary supplementation with the soybean and fatty acids has demonstrated to upregulate the expression of genes contributing to inflammatory responses as well as lipid and glucose profile. Hence, their selective inclusion/exclusion can be adopted as dietary treatment for IBD. Altogether, such immune-related gene-diet interactions might affect anthropometric parameters, metabolic profile, and cardiovascular measurements and thereby alter individual susceptibility to metabolic disorders (obesity, diabetes, and non-alcoholic fatty liver diseases), autoimmune disease (Crohn's disease), and cardiovascular diseases (atherosclerosis).

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Nutriepigenomic Immunity

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Key Points

- Epigenetic mechanisms and regulatory factors organize a flexible machinery by means of which multicellular organisms generate a heritable alteration in gene expression with respect to the fluctuating external environment.
- Epigenetic modifications by clever manipulation of maternal dietary and care can transpire independent of DNA sequences to shape a baby and future adult rich not only in immunity to physical diseases but also to brain and behavioral disorders.
- In addition to the role of hypothalamic–pituitary–adrenal (HPA) axis and adaptive transfer (breast milk or placenta) of immune factors, epigenetic mechanisms are of the main pathways involved in both maternal and neonatal nutrition-mediated immune programming.

Introduction

Sometimes, there is a commonsense link between nutrition and health – for example, eating too much correlates to obesity. Other times, the link is surprising – for example, when lean people get diabetes. Generally, both links need to incorporate the full range of genetic, environmental, and epigenetic factors. However, the role of genetic polymorphisms [1] and lifestyle factors is mainly expressed on the formation of the former link. On the other hand, epigenetic mechanisms including DNA methylation, chromatin variation, and non-coding RNA [2] and related regulatory factors such as methyltransferases (Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, Dnmt3L, and Dnmt1o), methyl binding proteins (MeCP2 and MBD1-4), and histone-modifying proteins (HDAC1 and Suvar39) [3] particularly contribute to the formation of the latter link. The epigenetic regulation of the link between nutrition and health starts from the preconception and gestation period and lasts throughout life [4].

Epigenetic events are defined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [5]. In fact, epigenetic regulatory factors, unlike genetic mechanisms, organize a flexible machinery by means of which multicellular organisms generate a heritable alteration in gene expression with respect to the fluctuating external environment [3]. There is a wide range of external stresses including radiation, pollutants, diet- and socio-economic status-related factors, trauma and injury, and temperature variation that have the ability to put the environment out of synch [6, 7]. When facing such an environment, that epigenetic flexibility might contribute to the development of responses which are not ubiquitously beneficial with possible consequences range from higher rates of point mutations and genomic instability to developmental abnormalities [8] and initiation of various diseases particularly obesity, diabetes, cardiovascular diseases, and cancers [3, 6]. On the other side, these stochastic reactions might arise from the body's repair system to precisely maintain gene–environment interactions as well as from the developing body to fulfil most perplexing functions, for example, brain programming (experience-dependent plasticity and synaptic transmission) [9] and metabolic programming (exercise-dependent plasticity) [10]. Suppose that if we are versed in the deliberate manipulation of the external environment, then that flexibility affords an opportunity for programming the genome under favored conditions.

What time is better than prior to and during gestation period? In addition, which treatment is easier than maternal diet design to guarantee the health of our kids? The *uterus*, where the fetus would be directly influenced by maternal diet, is the first environment human resides in, and in utero life probably is the most critical stage of the life cycle. The vision of geneticists for the future is the in utero treatment of some diseases by means of modifications at the DNA backbone. While right now epigenetic modifications by clever manipulation of maternal dietary and care can transpire independent of DNA sequences to shape a baby and future adult rich not only in immunity to physical diseases – particularly from chronic non-communicable (metabolic and cardiovascular diseases, cancers, and aging) diseases point of view [11, 12] – but also to brain and behavioral disorders [13, 14]. One implication of

the plasticity of epigenome programming is that some epigenetic marks are enduring so can transfer to the next generations [15] as well as some are reversible so allow re-programming after birth [16]. Further interesting is that the ability to shuttle back and forth between programming and re-programming the epigenome in a wanted direction will help the missed pieces of the puzzle of origin of human health become visible [17].

Flexible Nutriepigenomics: When Maternal Diet Immunizes the Offspring Against Immunological and Non-immunological Disorders

Epigenetic Programming of Offspring Genome by Maternal Diet: Evidence from Animal Studies

Methyl Supplements

The agouti (A) is among genes that control the distribution of pigment in the mouse hair. Some alleles (mutations) and related phenotypes of the gene are A (the phenotype agouti is characterized by "a band of yellow on the otherwise dark hair shaft"), A^{Y} (the lethal yellow is characterized by pure-yellow shaft), A^{VY} (the viable yellow is characterized by coat color variation with respect to epigenetic marks), a (the phenotype nonagouti is characterized by lack of the yellow band and "so there is solid dark pigment throughout"), and a^{t} (the allele produces a yellow belly with dark pigmentation elsewhere) (An Introduction to Genetic Analysis. 7th edition). The genotype A^{VY} /a produces different color coat phenotypes with respect to epigenetic marks. Among which, the pseudoagouti phenotype demonstrates the best health status and longevity. In fact, the phenotype represents CpG methylation in the A^{VY} gene which, in turn, correlates to lower ectopic expression of agouti and obesity rates [18].

Studies have frequently evaluated the effect of maternal diet on the distribution of A^{VY} allele when A^{VY} /a mated to a /a mice. Standard methyl-supplemented (MS) diet before conception, during pregnancy, and after birth can control the distribution of offspring to the high eumelanin mottling [19]. Moreover, there were "almost" or entirely agouti coat color patterns among offspring of mice which underwent the 3SZM diet that "contains 3× as much methyl supplement as MS diet plus zinc plus methionine" [19]. The more the maternal methyl supplement, the more the DNA methylation occurs at the offspring A^{VY} gene region that includes IAP-LTR and agouti sequences and then the more the trend moves into the more agouti phenotypes, for example, pseudoagouti (brown), in the offspring [20, 21]. It is important to be noted that this effect of methyl supplements on the distribution of A^{VY} gene occurs when A^{VY} /a sires mated to a /a dams not when A^{VY} /a dams mated to a /a sires [15]. Such germ-line epigenetic effect shows great endurance so that it can present in the next generation [15].

Similarly, the $Axin^{Fu}$ gene exhibits different tail kinking phenotypes with respect to epigenetic marks. As compared to that of maternal control diet, MS diet prior and during pregnancy and lactation resulted in a tail-specific increase in the $Axin^{Fu}$ methylation as well as a 30% reduction in tail kinking in the $Axin^{Fu}$ /+ offspring [22].

More interestingly, studies prove that epigenetic changes within the A^{VY} gene, which, like nearly 4% of human genes, is a transposable element, can metastasis to the adjacent gene regions (PS1A) [21].

In the pediatric mice, administration of methyl supplements was not different from the control diet in terms of response to dextran sulfate sodium (DSS) colitis [23]. However, the offspring of mothers feeding methyl supplement for 2 weeks prior to mating and throughout pregnancy and lactation had more weight loss and showed an exacerbated response to DSS exposure as represented in increased colonic shortening than control offspring. Persistent changes in 155 intervals (hypermethylation of 59 intervals vs. hypomethylation of 96 intervals) linked to the diet. Given its association with autoimmune disease especially inflammatory bowel disease (IBD), among which the Ptpn22-associated SmaI/XmaI interval was selected, and its hypomethylation was validated through a confirmatory study. On the gene expression study, the expression of more than 400 and 500 transcripts increased and decreased. Among which, decreased expression of the gene Cpn2 was validated. The microbiota was also altered in the MD

offspring. The most different genera were *Clostridia* and *Lactobacilli* with the average presence of 57% and 2.5% in the MD offspring vs. 27% and 46.5% in the control offspring.

The authors in the study [24] investigated the effect of maternal choline supplementation (from 2 weeks before mating and throughout mating and pregnancy) on the hepatic steatosis in the toxic milk (tx-j) model of Wilson's disease. Analysis of the fetal liver revealed lower hepatic content of copper in the tx-j offspring than in the control offspring, while there were higher hepatic concentrations of copper in the tx-j offspring at 21-day weaning. Maternal choline supplementation was found to further reduce copper levels in the fetal liver as well as to increase copper levels in 21-day offspring. However, it demonstrated no effect on histological changes in the fetal liver. In addition, maternal choline supplementation was associated with higher global DNA methylation in 21-day tx-j offspring. Maternal choline supplementation was able enough to restore reduction of the hepatic expression of genes related to methionine metabolism, cell cycle, and lipid metabolism, e.g., Mtr, Mat2a, Dnmt1, Dnmt3a, Sahh, Srebp1c, Grp78, Cpt1A, Pparα, and cyclin D1 in tx-j offspring.

The effect of maternal BPA exposure on the distribution of offspring coat color to yellow phenotypes vanished when methyl supplements were included in the maternal diet [25]. In this manner, the incidence of yellow phenotypes was not different between control offspring and BPA-exposed/methyl donor-supplemented and BPA-exposed/genistein-supplemented offspring (10–13%). Further, methyl supplements were able enough to nullify the negative effect of BPA on the CpG methylation at the A^{vy} IAP.

Genistein

Inclusion of genistein into the maternal BPA-exposed diet could not only avoid the overpresentation of yellow phenotype (unmethylated) among the offspring but also could maintain CpG methylation at the A^{vy} IAP similar to that was observed in the control offspring [25].

Consistently, maternal genistein-supplemented diet demonstrated to more distribute the offspring coat color to the pseudoagouti phenotype than the control diet (50% vs. 23%) [26]. The effect accompanied with an increase in the average percentage of cells methylated in CpG sites in the cryptic promoter region of the A^{vy} IAP. The most hypermethylated was CpG site 4. As expected, the pseudoagouti phenotype (methylated) was associated with noticeably lower mean week-60 body weights of 36.2 g in comparison with other phenotypes (54 to 59.5 g). At 60 weeks of age, there was a more than two-fold increase in the rate of normal weight among genistein-supplemented offspring than genistein-unsupplemented offspring (23% vs. 10%).

Adult (12 weeks of age) mice exposed to prenatal genistein (270 mg/kg feed) from 3 days before conception throughout pregnancy had an increase in numbers of RBCs and reticulocytes as well as in the mean corpuscular volume, red blood cell distribution width, and hemoglobin and hematocrit levels [27]. They also had higher numbers of pyrenocytes and granulocytic cells (neutrophils and eosinophils) in the bone marrow and peripheral blood, respectively. Microarray analysis revealed that 20% of the annotated genes are differentially expressed in bone marrow cells of adult offspring exposed to prenatal genistein. Gene ontology classified those genes mainly in the estrogen receptor signaling and hematopoiesis pathway. Favorably, prenatal genistein led to a reduction in the expression of genes related to the NF-Kb pathway (bcl2l1, cyclin D1, and Icam-1) and adhesion molecules (Ncam1, Cdh1, Intgb1, Itga1, MCP-1, P-selectin, P ligand, Nocht1, Mdm2, and Pdkcd). Consistently, the bone marrow of adult mice exposed to prenatal genistein revealed a modest increase in the methylation of repetitive DNA elements.

High-Fat Diet

As compared to the low-fat diet (5% fat and 70% carbohydrate), high-fat diet (40% fat and 35% carbohydrate) after weaning increased body weight at 12 months of age in that generation of Sprague-Dawley (SD) rats [28]. High-fat diet during pregnancy and lactation led to the altered food preferences so that the offspring tended to prefer foods high in fat and sugar [29]. The offspring showed an overall decreased methylation as well as decrease in the methylation of promoters related to the dopamine and opioid-related genes such as DAT, MOR, PENK, and POMC in

the specific brain regions. Consistently the expression of the aforementioned genes increased.

Strikingly, the effect of maternal high-fat diet on body size remained only for the third generation (F3) of female offspring from the paternal lineage [30]. It is consistent with gene analysis pattern that the imprinted genes from a paternal transmission, unlike maternal transmission, are liable to variation in the F3 female offspring. More clearly, neither the effect transmitted from the maternal lineage nor the male offspring represented the effect from both lineages. The F1 and F2 generations had impaired glucose tolerance and insulin sensitivity (as measured by GTT and ITT), while the F3 male offspring showed improvement in both of them. The study proves that imprinted loci play an important role in epigenetic programming.

The study [28] included some experiments where the offspring of high- and low-fat parents were randomly assigned to high-fat and low-fat diet during periconceptional period, pregnancy, and lactation (3–17 weeks of age). All the experiments revealed that, without respect to whether or not they fed high- or low-fat diet, the male offspring of high-fat parents gained greater weight and showed increase in the plasma levels of insulin and leptin and in the gene expression levels of lipoprotein lipase and leptin in the perirenal adipose.

The study [31] evaluated the epigenetic effects of low-fat, adequate-fat, and high-fat diet with fresh oil or butter on the offspring. As compared to the offspring of other maternal diets, the plasma proportions of omega-3 fatty acids (20:5n-3 and 22:6n-3) increased in the offspring of mothers feeding high-fat diets, especially high-fat fresh oil. However, there was a reduction in the hepatic proportions of 20:4n-6 and 22:6n-3. The analyses indicate that the higher the maternal diet is fatty, the less the hepatic mRNA expression of Fads2 in the offspring. Overall, the amount of fat in the maternal diet was positively associated with methylation at specific sites (CpG-623, CpG-394, CpG-84, and CpG-76) in the Fads2 promoter, and the association was more pronounced in the offspring of mothers feeding high-fat fresh oil diet. In particular, methylation at CpG-394 negatively correlated with the mRNA expression of Fads2 and the proportions of 20:4n-6 and 22:6n-3 in liver PC and PE and in plasma PC in male and female offspring. Of note, the diet change nullified related epigenetic changes.

As expected, the high-fat diet (45% fat) animals had higher weight and blood concentrations for glucose, leptin, and insulin than the low-fat (12%) ones [32], while the F1 and F2 offspring showed lower leptin concentrations in plasma. The transmission of longer body length and lower insulin sensitivity (as measured by ITT) through both F1 and F2 generations was confirmed for both male and female offspring. However, the increased body weight merely passed to the first, but second, generation. Surprisingly, the trait adiposity was present in none of first (F1) and second (F2) generations of high-fat diet parents. Although the hepatic expression of IGFBP-3 transcript increased in both male and female offspring of the F1, there was a female-specific pattern for the effect of maternal high-fat diet on higher plasma levels of IGF1 and hypothalamic expression of GHSR in both F1 and F2. Also, lower expression levels of GHSR transcriptional repressor (AF5q31) were found in the F1 female brains. Analysis of the GHSR promoter in F2 offspring revealed reduction in methylation at position – 72 in males and – 31 in females.

When compared to the control diet (12% fat), the maternal high-fat diet (62% fat) resulted in greater weight gain, higher blood levels of triglyceride and leptin, and lower blood adiponectin levels in both pregnant mothers and their offspring [33]. At 24 weeks of age, the high-fat offspring had higher SBP and impairment in glucose tolerance and insulin sensitivity than their control counterparts. The high-fat offspring also showed increase in the expression of leptin increased in the white mesenteric adipose tissue as well as decrease in the expression of adiponectin at 2, 12, and 24 weeks of age. Consistently, significant epigenetic changes like decreased H3K9 acetylation in the adiponectin promoter, increased dimethylation of H3K9 in the adiponectin promoter, and increased monomethylation of H4K20 in the leptin promoter were found in the high-fat offspring.

In the study [34], the offspring of rats exposed to paternal high-fat (HF) diet were assigned to high-fat diet (HF/HF) or control diet (HF/C) for

12 weeks after birth. Rats in the HF/HF group had higher body weight and lipid accumulation in the liver than those in the HF/C group. They also showed an elevated level of NAD+. When compared to the HF/C offspring, there was increase in the hepatic methylation of genes related to type 2 diabetes, arrhythmogenic right ventricular cardiomyopathy, phosphatidylinositol signaling system, adherens junction, axon guidance, adipocytokine signaling pathway, endocytosis, and cardiac muscle contraction in the HF/HF offspring. Consistently, the expression of genes including Adipor1, Cpt1a, Ppara, Rxra, Rxrb, Tnf, Tnfrsf1b, and Traf2 increased in the HF/HF offspring. However, there was a reduction in the expression of some genes, e.g., Mttp, Acacb, Fasn, Gpam, and Mapk8.

An elevated methylation of the maternally imprinted gene Peg3 was evident in spermatozoa of offspring of mothers who fed high-fat diet for 12 weeks prior conception and throughout pregnancy as compared to the male offspring of control mice [35]. On the other hand, there was a significant reduction in methylation of the paternally imprinted gene H19 in spermatozoa of offspring of diabetic mothers (who received a streptozotocin injection 15 days prior to mating) compared to that of nondiabetic mothers.

The study [36] included three dietary groups of female mice: control, HF diet (obese), and HF diet switched to a control diet 2 months before conception (weight loss). More than 25% of the offspring of mothers in the HF group had growth problems, while none of the fetuses in the weight loss or control group experienced growth restriction. As compared to the offspring of mothers who fed a control diet, the offspring exposed to maternal obesity showed differential expression of 19 genes in the liver or placental labyrinth, while there were only seven genes with an altered expression in the offspring exposed to maternal weight loss. This indicates that maternal weight loss prior mating might help to restore obesity-related changes in the offspring epigenome. Maternal obesity was associated with upregulation of genes (writers, KATs; erasers, HDACs; and readers, Brd2) involved in histone acetylation in the fetal liver. Particularly, weight loss could normalize the expression of KATs and showed a trend toward restoration of HDACs as well.

Maternal HF diet demonstrated to reduce by greater than 70% the gene expression and protein content of $P16^{INK4\alpha}$ while increasing the number of mammary cells arrested in S-phase in the offspring mammary glands [37]. Consistently, the HF offspring showed histone modifications including a reduction of acetylation of histone H4 (H4Ac) at the $P16^{INK4\alpha}$ promoter and related CpG-rich sites and increase in the HDAC1 levels and in the HDAC3 interaction within the p16INK4a promoter. In this manner, the offspring exposed to maternal HF diet are more liable to cancer.

The study [38] demonstrated that early exposure to HF diet would affect the offspring circadian system. Compared to the control offspring, there was more between-individual variation in the overall mRNA copy number of circadian genes including Npas2, Per2, and Rev-erb-α in the HF offspring, while the overall phase difference was decreased in the HF offspring. Postweaning high-fat diet increased the hepatic expression of Npas2 and that this increase was found to be due to histone modifications (increase in H3K14ac occupancy at the RORE promoter region and in the first intron of Npas2) rather than due to methylation changes.

High-Fiber Diet

Maternal high-fiber diet demonstrated to dramatically change the offspring microbiota as represented in high levels for short-chain fatty acids (SCFAs) and acetate in serum and feces and high propionate levels in the serum [39]. However, no-fiber diet was associated with higher Shannon and chao1 indices in the offspring. Intestinal microflora was dominated by the phylum *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the high-fiber, no-fiber, and control diets correspondingly. In parallel, experiments revealed that high-fiber diet and acetate for 3 weeks impede the induction of allergic airways disease (AAD). The offspring (3–16 weeks of age) of mothers who underwent high-fiber diet or acetate consumption during pregnancy appeared immune to

AAD as reflected in lower numbers of immune cells (particularly eosinophils) in the bronchoalveolar lavage fluid (BALF) and blood, lower levels of Th2 cytokines and IgE, and limited lung inflammation. More interestingly, this immunity seems to transfer in utero because it was not found in the offspring of mothers who fed the aforementioned diets after birth and during lactation.

SCFAs play an important role in fostering the homeostasis of T regulatory cells in the colon possibly through the inhibition of HDAC6 and HDAC9 [40]. Like HDAC9 deficiency, acetate consumption has shown to inhibit HDAC activity and thereby make mice immune to AAD. A key difference in their action was that the inhibitory effect of acetate consumption on HDAC activity did not transfer to the offspring, but the effect of HDAC9 deficiency did [39].

Acetate is protective against AAD because it is in aid of T regulatory cells. For both adults and their offspring, acetate consumption during pregnancy could bring about higher acetylation at H4 in the Foxp3 promoter region, higher Foxp3 levels in the lung tissue, and finally higher number and function of Tregs in peripheral lymph nodes [39]. Of note, lung tissues revealed lower expression of three genes Nppa, Ankrd1, and Pln for both the offspring of mice treated with high-fiber diet or acetate [39].

Protein-Restricted Diet

As summarized, low-protein diet during pregnancy and lactation leads to increased methylation at the promoter site and histone modifications that would lapse into a considerable silencing of promoter-enhancer interaction and thereby lowering the mRNA levels of Hnf4a [41]. Further interesting is that ageing by its own can negatively affect the expression of Hnf4a by epigenetic mechanisms as low-protein diet does.

By contrast, subsequent to epigenetic changes (decreased methylation at the promoter sites, decreased expression of MeCP2 and Dnmt1, and histone modifications), the mRNA expression of genes PPAR-α and GR and their targets AOX and PEPCK increased in the liver [42, 43]. Additionally, less than 40% of CpGs surrounding the promoter region of ACE-1 in the brain were methylated in the maternal low-protein diet (MLPD) offspring, while almost 80% showed methylation in the control offspring [44]. Consequently, the mRNA levels of angiotensinogen and ACE-1 increased in the brain of MLPD offspring, while both the mRNA levels and protein content of AT2 receptors reduced in the brain [44].

When compared to the control diet (180 g/kg casein) offspring, protein-restricted diet (90 g/kg casein) was associated with a global reduction in the methylation at CpG sites in the PPAR-α promoter as well as with lower methylation at the individual CpG sites 2, 3, and 16 [45]. Further, there is an inverse relationship between the mRNA expression levels of PPAR-α and methylation at CpG sites 3 and 16.

The epigenetic changes of maternal protein-restricted diet during pregnancy in the F0 generation including a reduction in the methylation of the $GR1_{10}$ and PPAR-α promoters were observed in both F1 and F2 male offspring [46]. The only gene significantly altered was PEPCK which increased in the liver of the F1 and F2 generations.

Overall, maternal low-protein diet was associated with lower body weight [44, 47] and lower serum levels of lactic acid [47]. When compared to their counterparts, low glucose in serum and high hepatic G6PC enzyme activity was only found among offspring males, not females. Also, the effect of diet on increasing hepatic mRNA expression of fructose-1,6-bisphosphatase 1 (FBP1) and glucose-6-phosphatase and catalytic (G6PC) was specific to offspring males. Although the increase in mRNA expression and protein concentrations of GR was evident for both offspring males and females, high GR binding to fragments 1 and 2 was specific to offspring males, while GR binding to fragment 3 increased in offspring females. Consistently there were gender-specific epigenetic changes.

Maternal low-protein diet was significantly related to lower body weight and body fat and smaller adipocyte size in the offspring at 3 months of age [48]. Microarray analysis of the adipose tissue indicated higher expression of only one

microRNA in the low-protein offspring at both points of time (22 days and 3 months of age). Interestingly, that microRNA was miRNA-483-3p, which its higher expression in adipose biopsies was previously associated with low-birth weight (LBW) in humans. Further, the protein content of GDF3, a target of miRNA-483-3p, decreased in both the rat adipose tissue and the adipose tissues related to LBW men.

Maternal protein restriction (for 7 weeks during pregnancy) noticeably altered the behavioral profile of offspring, as reflected in increased fear/anxiety-like, novelty seeking, and prosocial behaviors [49]. Genome-wide DNA methylation analysis identified more than 550 hidden Markov model (HMM) regions, which were differentially methylated in the offspring of mothers who fed protein-restricted diet as compared to those of mothers who received control diet. Consistently, maternal protein restriction resulted in differential expression of 80 genes in the offspring' brain.

The study [50] showed that the effect of maternal protein restriction/excess on the expression of genes involved in DNA methylation and methionine metabolism (DNMT1, DNMT3a, DNMT3b, BHMT, MAT2B, and AHCYL1) differs with the age of offspring. This was true about hepatic global DNA methylation, which at gestational day 95 was significantly decreased in the liver of offspring exposed to maternal protein restriction than in that of offspring exposed to control maternal diet. Similarly, at postnatal day 28, there was lower DNA methylation in the offspring exposed to maternal protein excess compared to those exposed to control maternal diet. However, no difference in global DNA methylation was found between dietary groups at other time points (birth and postnatal day 188). Moreover, fetuses exposed to maternal protein restriction displayed a lower hepatic expression of NCAPD2, NCAPG, and NCAPH compared to those exposed to control maternal diet. The expression of these genes was decreased with age.

Protein restriction during pregnancy led to reduction of hepatic expression of Gnas in offspring at week 3 after birth and this reduction persisted until 12 weeks of age [51], while protein restriction during lactation was associated with an increased expression of Grb10 and Pparα at week 3. However, offspring exposed to protein restriction during lactation showed lower expression of Pparα at week 12. This indicates that the impact of maternal dietary on the offspring genome is at least in part regulated by developmental period. Maternal protein dietary was found to significantly affect DNA methylation at the imprinted genes. However, gene-specific analysis indicated higher methylation at Pparα at week 3 in offspring exposed to protein restriction during lactation.

Folic Acid Supplements

These supplements are apparently able enough to nullify the epigenetic alterations induced by the protein-restricted diet and therefore the increased expression of mentioned genes [42, 43, 45]. As compared to the offspring of control diet (180 g/kg casein) or protein-restricted diet (90 g/kg casein) plus 5 mg/kg folic acid, inclusion of 5 mg/kg folic acid to the maternal protein-restricted diet enhanced methylation at CpG sites 5 and 8 in the PPAR-α promoter [45]. However, folic acid supplementation could not reverse the negative impact of maternal protein restriction on the behavioral phenotype of offspring [49].

In addition, the gene expression pattern of cerebral tissues from offspring was found to be influenced by the doses of folic acid in maternal diet and that the effects were sex-dependent [52]. There was a reduction in the cerebral expression of transcription factors, e.g., Nfix, Runx1, and Vgll2, in male offspring of mothers who fed high doses of folic acid (20 mg/kg) when compared to their counterparts of mothers who fed custom doses of folic acid (2 mg/kg). Nevertheless, female offspring exposed to high maternal folic acid (HMFA) showed elevated expression of these transcription factors in comparison with female offspring exposed to control maternal diet. However, both male and female offspring exposed to HMFA had lower expression of DNA methyltransferase Dnmt3b, but not Dnmt3a. Among the imprinted genes investigated, there was an increase in the expression of Dio3 in male offspring and in the expression of H19 and Xist in female offspring exposed to HMFA. Of note was elevated expression of susceptible genes

(Auts2 and Fmr1) for autism in female offspring whereas a reduced expression of these genes (Auts2) in male offspring.

Betaine-Supplemented Diet (BSD)

The offspring of sows feeding BSD had higher serum concentrations of betaine, lactate, and glucogenic amino acids (serine, glutamate, methionine, and histidine) [53]. They also showed increase in the hepatic content of betaine, glycogen, BHMT, AHCYL1, MAT2B, PEPCK2, FBP1, and PC as well as in the mRNA expression of BHMT, AHCYL1, PEPCK2, and FBP1 in the liver. The enzyme activity and protein content of PEPCK1 were also increased in the liver whereas its mRNA levels decreased. Further, the expression of miRNAs that are known to control the expression of PC (miRNA-184 and miRNA-196b) and PEPCK1 (miRNA-1403p, miRNA-424-3p, miRNA-196b, miRNA-370, miRNA-30b-3p, and miRNA-92b-5p) at the post-transcriptional level were decreased in the liver. Consistent with changes in protein contents and mRNA expression levels, there was a reduction in the methylation on the promoter of PEPCK2 and FBP1 genes. By contrast, there was increase in the methylation at the PEPCK1 promoter; repression mark H3K27me3 on the promoter of PEPCK1; activation mark H3K4me3 on the promoters of PEPCK2, FBP1, and G6PC; and lysine methyltransferase SETD7. Further GR binding to PEPCK2 and G6PC gene promoter increased.

Methyl-Deficient (MD) Diet

Periconceptional maternal MD diet led to the nonclinical laboratory changes in micronutrients such as lower levels for vitamin B12, folate, and methionine in plasma and higher levels for homocysteine in plasma, ovarian follicular fluid, and granulosa cell lysates [54]. The MD offspring at both measures at 3 and 22 months of age had greater weight gain than control offspring. There was a male-specific effect of maternal MD diet on the acute-phase response to ovalbumin, body composition, and cardiovascular function in the offspring. Compared to control males, MD males showed an exacerbated acute-phase response at 12 months, were fatter at 22 months, and had higher arterial blood pressure and systolic, diastolic, and mean arterial pressure responses at 23 months. Regarding glucose metabolism, the MD offspring, particularly males, demonstrated greater insulin response to i.v. glucose infusion. Restriction landmark genome scanning led to the identification of 71 loci, which differently methylated in MD and control offspring. Interestingly, maternal MD diet led to the unmethylation or hypomethylation at nearly 90% of these loci, and, more interestingly, there was a fourfold increase in the number of male-specific than female-specific altered loci (53% vs. 12%). This is consistent with more significant effects of maternal MD diet on the offspring males than females.

The study [55] investigated the effect of maternal methyl-donor-deficient diet (MDD; a diet without vitamin B12 and folate) on the offspring genome and epigenome. There was a reduction of body weight as well as increased liver/body weight ratio in MDD offspring compared to control offspring. In addition, MDD offspring had higher levels of fatty acids, cholesterol, and triglyceride. Analysis of liver transcriptome indicated altered expression of more than 6000 genes in MDD offspring. The affected genes were mainly associated with metabolic processes such as lipid metabolism, ER stress, mitochondrial function, glucose and cholesterol metabolism, blood coagulation, and iron homeostasis. Pathway analysis also remarked the PPAR signaling pathway as top affected pathway. Consistently, the hepatic methylation of more than 1000 genes mainly associated with fatty acid and lipid metabolism was altered by MDD. More interestingly, there was overlap transcriptome and methylome for 266 genes, named "master" genes, which are among genes contributing to the renin-angiotensin system, phospholipid homeostasis, and mitochondrial metabolism. Of note is the association of these genes with non-alcoholic fatty liver disease (NAFLD).

Vitamin A-Deficient Diet

Maternal vitamin A deficiency (VAD) prior mating and during pregnancy resulted in the serious cardiac defects [56]. Supplementation during pregnancy (VADS) could reduce malformations, but it was not able to eradicate them. Analysis of

the offspring hearts revealed that maternal vitamin A deficiency could induce an increase in methylation percentage in the CpG loci of GATA-4. Consistently, the cardiac expression of GATA-4 was reduced in the VAD offspring. VAD offspring also displayed higher expression of DNMT1 and lower expression of DNMT3a and DNMT3b.

Bisphenol A (BPA)

Despite no difference in global methylation between exposed and unexposed groups, maternal exposure to BPA led to an increase in the mean methylation percentage in the offspring tail and more precisely at the $Cabp^{IAP}$ epiallele [57]. There was a dose-dependent relationship between maternal exposure to BPA and the coat color in the offspring. As compared to the unexposed offspring, the offspring exposed to 50 mg BPA/kg, 50 μg BPA/kg, and 50 ng BPA/kg exhibited an increase in the exhibition of yellow, pseudoagouti, and slightly mottled and slightly mottled and heavily mottled phenotypes.

Similarly, addition of 50 mg BPA/kg to the maternal diet resulted in the distribution of offspring coat color to the yellow phenotypes [25]. As compared to BPA-unexposed offspring, there was a significant reduction in the average percentage of cells methylated at nine CpG sites in the cryptic promoter region of the A^{vy} IAP in the BPA-exposed offspring. Further interesting was that the association of maternal BPA exposure and the offspring coat color did not remain significant when considering the effects of BPA on methylation. This clearly asserts that what plays an important role in distribution of coat color by BPA exposure is methylation in the A^{vy} IAP promoter. The BPA-exposed offspring also demonstrated decrease in methylation of CpG sites 6–9 in the Cabp IAP gene.

In utero exposure to 50 mg BPA was associated with liver CpG methylation at the Glcci1 Repeat 1 locus as well as with CpG methylation at the first and fifth CpG sites in Glcci1 Repeat 2 [58].

Nicotine Exposure

No significant change in body weight measurements, food intake, locomotor activity, energy expenditure, fasting glucose measurements, and glucose tolerance test was observed between nicotine-exposed and nicotine-unexposed offspring [59]. However, nicotine exposure for 4 weeks prior mating and throughout pregnancy improved the action of leptin on melanocortinergic pathways contributing to body-weight regulation in the hypothalamic arcuate proopiomelanocortin (POMC) neurons. This improvement might be underpinned by altered expression of long noncoding RNAs (lncRNAs), which, in turn, demonstrated to affect the expression of genes contributing to a variety of signaling pathways particularly opioid proopiomelanocortin, enkephalin release, and nicotinic acetylcholine receptor.

Vitamin D

Both G1 and G2 generations displayed the negative impact of maternal vitamin D depletion (for 5 weeks before mating and throughout gestation and weaning) on the body weight pattern [60]. The study showed that vitamin D depletion decreases DNA methylation at paternal (H19/Igf2 and Dlk1/Meg3) and maternal (Snrpn and Grb10) imprinted loci. More interestingly, these methylation changes were corresponded to body weight measurements.

Multivitamins

In the study [61], rats received a control diet or a high-fat soluble vitamin diet (HFS; tenfold vitamins A, D, E, and K). There was no difference in body weight, food intake, and oil preference between offspring exposed to HFS and those from control diet. However, sucrose preference was reduced in HFS offspring compared to control offspring. Analysis of brain gene expression at birth revealed reduction of hippocampal expression of genes involved in dopaminergic pathways Drd5 and Dat in HFS offspring. However, neither global DNA methylation nor gene-specific DNA methylation at Drd5 and Dat was not different at birth in HFS offspring than control offspring.

Different Diets

Study in sheep [62] showed higher content of IGF2 and lower content of its receptor IGF2R in perirenal fat of the offspring of mothers fed corn (CN: starch) during mid- to late-gestation as compared to that of offspring of mothers feeding alfalfa haylage (HY: fiber) or dried corn distiller's grains (DG: fiber plus protein plus fat).

However, the expression of growth factor receptor-bound protein 10 (GRB10) increased in the DG offspring. There was higher expression of CEBPA expression in the subcutaneous adipose tissue from the offspring of HY diet as compared that of other two diets.

In the study [63], there were five experimental diet groups: normal, HF diet, 50% germinated brown rice (GBR), low dose oryzanol extract (LOE), and high dose oryzanol extract (HOE). The level of DNA methylation increased in the offspring of dams who fed HF diet as compared to those in other experimental groups (GBR, LOE, and HOE), while the offsprings of the HOE dams showed an elevated total histone H3 acetylation and a reduction in H4 acetylation than those of the HF dams. When compared to the offspring of other dietary groups, the offsprings of dams who received HF diet had higher levels of insulin, 8-iso-prostaglandin, RBP4, and leptin and lower levels of adiponectin in serum.

In the study [64], the authors investigated the effect of a double-insult dietary intervention (folate depletion from 4 weeks prior mating throughout pregnancy and weaning and high-fat diet from weaning) to the brain in the adult offspring (6 months of age). Maternal folate depletion led to an increase in the BER-related incision activity in offsprings' brains at weaning, whereas exposure to HF diet from weaning resulted in a reduction of BER-related incision activity. Feeding a HF diet was found to decrease the expression of Neil1 and Mutyh in offsprings' brains. Gene-specific methylation indicated the effect of diet on the gene Ogg1 in terms of higher methylation with maternal folate depletion and lower methylation with HF diet from weaning.

Maternal diet lacking folate and vitamin B12 (from 1 month before pregnancy until postnatal day 21) led to loss of body weight, reduction of folate and vitamin B12 levels in blood, and decreased S-adenosylmethionine/S-adenosylhomocysteine ratio in the cerebellum while increasing the cerebellar content of homocysteine, methylmalonic acid, and succinic acid in the offspring [65]. At postnatal day 19–21, the linear walking was worsened in the MDD offspring compared to the control offspring. Reduction of the expression of synaptic proteins synapsins I and II was observed only in female offspring, whereas there was lower content of postsynaptic density protein 95 in both male and female offspring. Surprisingly, methylation of syn genes was not influenced by MDD. Further proteomic and epigenomic analyses revealed that reduction of synapsins might be due to reduced methylation of ER-α, whereby diminishing the interaction between ER-α with Src and therefore the activity of ER-α pathway in neuroprogenitor cells.

Epigenetic Programming of Offspring Genome by Maternal Behavior: Evidence from Animal Studies

CRF is required for the HPA to respond stress. Lower CRF levels result in an impaired response to stress, and therefore behaviors such as pup licking/grooming and arched-back nursing (LG-ABN) begin to display. The offspring of LG-ABN mothers also represent a blunted stress response system. Maternal behaviors cause this effect through epigenetic mechanisms. Further interesting is that intracerebroventricular infusion of methyl supplements to adult offspring (3 months old) could drive these mechanisms into the reverse direction. In addition to epigenetic changes including a reduction in methylation at the hippocampal exon 1_7 GR promoter and binding of NGFI-A protein to the hippocampal exon 1_7 GR promoter, MS could lessen mRNA and protein concentrations of hippocampal exon 1_7 GR, while it promoted corticosterone responses to stress [16].

Compared to those reared by low-linking mothers, global histone-H4 acetylation in the olfactory bulb increased in rats reared by high-licking mothers [66].

Epigenetic Programming of Offspring Genome by Maternal Diet: Evidence from Human Studies

Anthropometric Measures and Blood Pressure

These studies prove the association of altered methylation at regions related to the genes GR and 11βHSD2 with neonatal and adult anthropo-

metric measures and more interestingly with blood pressure [67]. Greater consumption of meat, fish, and vegetable and lower consumption of bread/potato in late pregnancy (>20 weeks) might affect methylation at GR exon 1F and CpG sites in 11βHSD2, which, in turn, would influence systolic and diastolic blood pressure [67].

Study of DNA methylation in umbilical cord tissues revealed the association of increased methylation at RXRA and eNOS sites with the child's adiposity and BMI at 9 years of age [68]. While methylation at SOD1 negatively correlated to child's trunk-to-limb fat ratio. More than 25% of the fat mass variation was attributable to changes in the RXRA methylation and gender. Further interesting is the association of higher RXRA methylation with lower maternal carbohydrate intake, which, in turn, was related to higher neonatal adiposity. Negative correlations existed between methylation at three sites related to PIK3CD and SOD1 genes and birthweight.

Asthma

Studies of pregnant women revealed direct association between fiber consumption during late pregnancy and serum acetate levels, and more interestingly children of women with acetate levels equal to or above the median were less likely to consult a general practitioner for respiratory problems (cough or wheeze) in the first year of life [39].

Choline Intake

The authors in [69] investigated the effect of maternal high-choline intake (930 mg/day) for 12 weeks during the third-trimester pregnancy in comparison with adequate choline intake (480 mg/day). Several epigenetic changes were found. The high-choline placental tissues revealed increase in the global DNA methylation as well as in the average CpG methylation of the CRH and NR3C1 promoter regions. However, there was a reduction in the methylation at CpG unit C of GNAS-AS1 in placenta and average CpG methylation of the CRH and NR3C1 promoter regions in cord leukocytes. Further, the gene expression of CRH and EHMT2 decreased in placenta tissue.

The study [70] investigated the effect of intake of methyl-group donors during preconception, each of the three pregnancy trimesters, or lactation on gene-specific (DNMT1, IGF2 DMR, RXRA, and LEP) buccal DNA methylation in 6-month-old infants. Before pregnancy, high maternal folate intake correlated to increased methylation at RXRA and decreased methylation at LEP. Betaine intake was associated with increased methylation at RXRA as well, while the study found a link between maternal folic acid consumption and lower methylation of IGF2 DMR. Folic acid intake during the second trimester was associated with decreased methylation at DNMT1, whereas choline and folate intake during the third trimester was shown to be associated with an increased methylation at DNMT1. Infants of mothers with high choline intake during lactation displayed higher methylation of RXRA.

Mediterranean Diet Adherence

The Newborn Epigenetic Study [71] investigated the possible impact of Mediterranean diet adherence on DNA methylation in infants at birth. Women and their infants were categorized into low, medium, and high Mediterranean adherence. Overall, infants from mothers with low Mediterranean adherence were more likely to have lower methylation at the MEG3-IG region. Interestingly, the association was confirmed among girl not boy infants. Infant methylation at the NNAT and MEG3 regions was also affected by Mediterranean diet adherence during pregnancy. Among these associations, only the association between Mediterranean adherence and methylation at the MEG3-IG region among girl infants remained significant after adjustment. In addition, low adherence to a Mediterranean diet appeared to increase methylation at the PLAGL1 and H19 DMRs in boy infants in adjusted models.

Alcohol and Vitamin B12 Intake

A cohort study [72] of 254 mother-child pairs revealed ZAC1 DMR methylation and fetal and infant anthropometric measures. In addition, there was a correlation between ZAC1 DMR methylation and C peptide levels in cord blood.

More interestingly, ZAC1 DMR methylation was positively associated with Vitamin B2 (riboflavin) intake prior pregnancy as well as with alcohol intake during the third trimester of pregnancy. However, neither maternal vitamin B9 intake nor folic acid supplementation influenced ZAC1 DMR methylation in children.

Cord blood LINE-1 methylation ($n = 516$) was negatively correlated with periconceptional betaine and cadmium intake [73]. However, there was a positive association between LINE-1 methylation during the first trimester and periconceptional cadmium intake. A negative association between cord blood LINE-1 methylation and periconceptional choline intake was also found in males.

Nutriepigenomic Immunity

As we discussed in Chap. 24, Nutrigenomic Immunity, dietary factors such as nutrients and fatty acids are known to affect the expression of genes and content of proteins contributing to immunity and inflammation, thereby altering the tissue microenvironment, cellular context, and the microbiome [74]. This alteration would pave the way for the development of non-communicable diseases favorably allergic and immune disorders. In addition to the role of HPA and adaptive transfer (breast milk or placenta) of immune factors, epigenetic mechanisms are of the main pathways involved in both maternal and neonatal nutrition-mediated immune programming [75]. Below is a brief discussion of how nutritional factors influence the immune system.

Figure 25.1 is a schematic of epigenetic mechanisms, e.g., DNA methylation and histone modifications contributing to the differentiation of T cells into Th1, Th2, Treg, and Th17 subsets [76]. The neonatal period is concerned with a reduced Th1 cell functionality as reflected in lower IFNγ expression while the postnatal period with Th1 cell maturation. The main epigenetic changes accompanying these developmental stages are hypermethylation and progressive demethylation of the IFNγ promoter, correspondingly [77]. A variety of nutritional factors counteracting Th1 cell maturation and thereby relatively aggravating Th2 cell responses have the potential to raise the risk of allergic diseases.

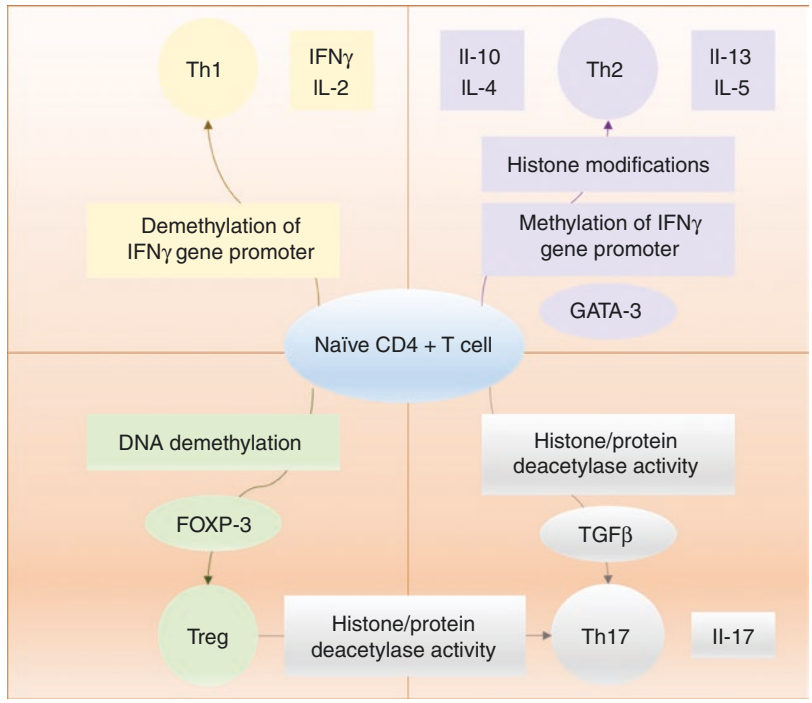

Fig. 25.1 Epigenetic mechanisms contributing to the differentiation of T cells into Th1, Th2, Treg, and Th17 subsets

DNA methylation is an epigenetic mechanism strongly affected by nutrients (folate, vitamins B2, B6, and B12, methionine choline, and betaine) [78], bioactive food components (tea polyphenols, genistein from soybean, isothiocyanates from plant foods, curcumin, and curcumin-derived synthetic analogs) [79], diet (fiber, protein, fat, and hormones) [80], ethanol, and carbohydrates [81]. As illustrated in Fig. 25.1, demethylation of IFNγ gene promoter contributes to the expression of Th1 cell cytokines (IFNγ and IL-2), whereas its methylation to that of Th2 cell lineage. DNA demethylation might also assist the expression of Th2 cell-related cytokines (IL-4, IL-5, IL-10, and, IL-13) and Treg cell-related regulators (FOXP3). Among the best-investigated micronutrients required for DNA methylation are folate, vitamin D, and zinc [79]. Low folate conditions have been correlated with the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) that may be unfavorable. However, folate supplementation has demonstrated to decrease DNA methylation and therefore increasing the expression of tumor suppressor genes. Changes in DNA methylation seem to lie behind the anti-inflammatory benefits of vitamin D – including potent inhibition of activating T cells (NFAT) transcription factors and reduction of IL-12 and IL-17 – to patients with multiple sclerosis (MS). Similarly low zinc can disturb DNA methylation patterns associated with immunity, inflammation, and related disorders, e.g., atherosclerosis and diabetes. Interestingly, under low folate, low methionine, or high homocysteine conditions, T cells from subjects aged 50 years or older demonstrated DNA demethylation and overexpression of KIR and TNFSF7 (CD70) genes, which have been associated with autoimmune diseases, in comparison with those from young adults [82]. It, therefore, is concluded that old patients are more vulnerable to the nutrient depletion, related epigenetic changes, and consequent diseases. Contrary to these lines of evidence is the exacerbation of inherited allergic disorders following maternal methyl donors supplementation [83].

Generally, histone modifications including histone acetylation, histone methylation, and histone biotinylation are likely to have a role in the development of Th2 and Th17 cell lineage. Particularly, histone deacetylase (HDAC) and histone acetyltransferase (HAT) are essential to maintain the histone acetylation balance that would regulate the expression of several inflammatory mediators, notably pro-inflammatory (IL-1β, IL-5, IL-6, IL-8, IL-12, and TNFα) and anti-inflammatory (IL-10) cytokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) and pathways (NF-κB). Nutritional factors that produce a change of histone acetylation in favor of anti-inflammatory effects include caloric restriction, vitamin D, resveratrol, allyl sulfur compounds, plant flavonoids (apigenin and chrysin), 6-shogaol, and curcumin. These nutrients appear potentially promising for the control of inflammation not only in peripheral inflammatory diseases (rheumatoid arthritis) but also in central inflammation-associated diseases (Alzheimer's disease) [79]. Histone methyltransferases and histone demethylases serve as controller enzymes for the equilibrium of histone methylation, which its inhibition by AdoMet can lead to inflammatory effects [80].

Chromatin remodeling occurs in two main fashions by ATP-dependent chromatin remodeling complexes and Polycomb repressive complex. The switch mating type/sucrose nonfermenting (SWI/SNF) is an ATP-dependent chromatin-remodeling complex that would help to change nucleosomal structure using free energy of ATP hydrolysis. Studies show the role of this complex in immune responses [80]. More precisely, the development of Th2 cell lineage is correlated with chromatin remodeling at the corresponding locus [77].

MicroRNAs organize another epigenetic mechanism through which nutritional factors including vitamins, fat feeding, protein, hormones, curcumin, and ethanol leave an effect on the immune system [79, 81].

In addition, the microbiota is an epigenetic trick to the maturation of the immune system [84] and particularly to the development of immune tolerance because of its collaboration with the Th1/Th2 balance [85]. Dietary components such as fat and fiber play the most important role in

shaping and timing the composition of the early microbiome [74], which can be transmitted through care giving, breastfeeding, direct intrauterine seeding, and passage through birth canal or C-section [86, 87]. Nutrition can push the gut microbiome into an inflammatory state by modulation of TLR4-mediated inflammation, alteration of immune cell function, and shift in local nutrients [87]. An early pro-inflammatory microbiome has the potential to actively participate in the pathogenesis of later developing infections; immunological disorders particularly asthma, allergies, and autoimmune disorders [88]; and inflammation-associated disorders, particularly cancers [87] and obesity [89]. For example, the study [90] found higher fecal *Clostridium difficile* load in children with a positive family history of food allergy. Since dietary patterns are directly involved in the dynamics of the gut microbiota, it is expected to see the link between dietary patterns and the risk of immune and inflammatory disorders. The Mediterranean diet during pregnancy and early childhood proved to lower the prevalence of atopy and wheeze [74], while Western diet may predispose individuals to infections, allergies, cancers, and autoimmune diseases [87]. Generally, all the macronutrients, e.g., salt, sugar, red meat, and saturated fats, that are mainly contained in the Western diet are capable to cause gut dysbiosis [87]. More precisely, salt leads to the upregulation of the IL-17-induced inflammation, which is implicated in the pathogenesis of autoimmune diseases [91]. Long chain polyunsaturated fatty acids (LCPUFA) cause inflammation through altering the concentration of inflammatory mediators (prostaglandins) and immunomodulatory factors (IL-10 and thymic stromal lymphopoietin) [74]. Studies report that consumption of n-3 PUFA correlates with an inhibition of TLR4 signaling and subsequent production of inflammatory cytokines (IL-1, IL-6, and TNFα) [87], as reflected in lower risk of allergies [74], whereas consumption of saturated fats and n-6 PUFA, a potential trigger for TLR4-induced inflammation, has been associated with higher risk of allergies. While restoring the microbiota profile that can be obtained using probiotics has the potential to alter the immune system response for preventing immune diseases [81], probiotics, for example, *Bifidobacterium* breve (DSMZ 20213) and LGG (ATCC 53103) might delay the progression of IBD by decreasing histone acetylation and increasing DNA methylation, which totally result in a reduced activity of IL-23/IL-17 axis [92]. Furthermore, SCFA which is an end product of the fermentation of probiotics correlates to epigenetic modifications [93]. Particularly, the SCFA butyrate functions as an HDAC inhibitor to impede inflammatory responses [93]. Nutrients including selenium, zinc, vitamin C, and vitamin E have also shown to influence the content of immunomodulatory factors (IL-12) with the potential for inflammation control and therefore combating allergic conditions [74]. On the contrary, these nutrients possess antioxidant features that promote Th2 cell differentiation [74], which is closely linked to allergy and asthma [94]. In particular, recent research identifies a significant positive association between vitamin D supplementation at 1 and 2 years of age and development of childhood asthma as demonstrated in a population-based birth cohort ($n = 910$) [95].

For the aging scenario, epigenetic changes also provide a mechanism underlying the effects of nutritional factors, e.g., methyl donors, fatty acids, vitamins, and phytochemicals, on the intracellular conditions, e.g., inflammation and stress, which in turn determine the metabolic state. Different chromatin modifying writer-eraser enzymes contribute to these epigenetic changes dependent on the type of metabolic state that may be glycolytic (sirtuin, PARPs, LSD1, HIFs, and P300) or oxidative (HATs, HMTs, DNMTs, HDMs, and TETs) [96].

Conclusions

The first part of the chapter presented evidences supporting that epigenetic modifications by clever manipulation of maternal dietary, and care can transpire independent of DNA sequences to shape a baby and future adult rich in immunity to physical diseases as well as to brain and behavioral dis-

orders. The second part listed epigenetic mechanisms that are involved in both maternal and neonatal nutrition-mediated offspring immune programming.

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