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

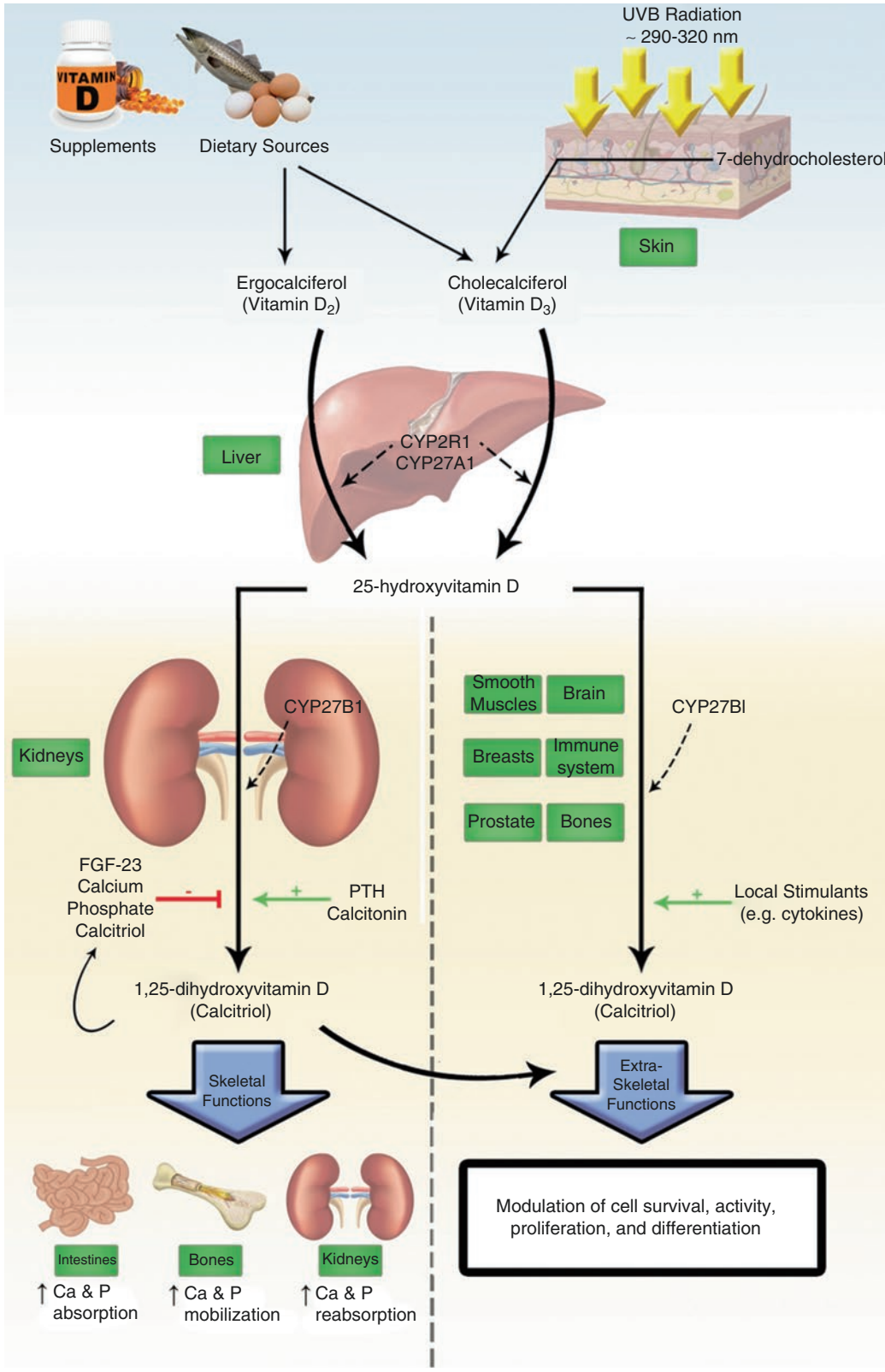

Fig. 2.1 Synthesis and metabolism of VitD. Ca calcium, P phosphate, PTH parathyroid hormone, FGF-23 fibroblast growth factor 23

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

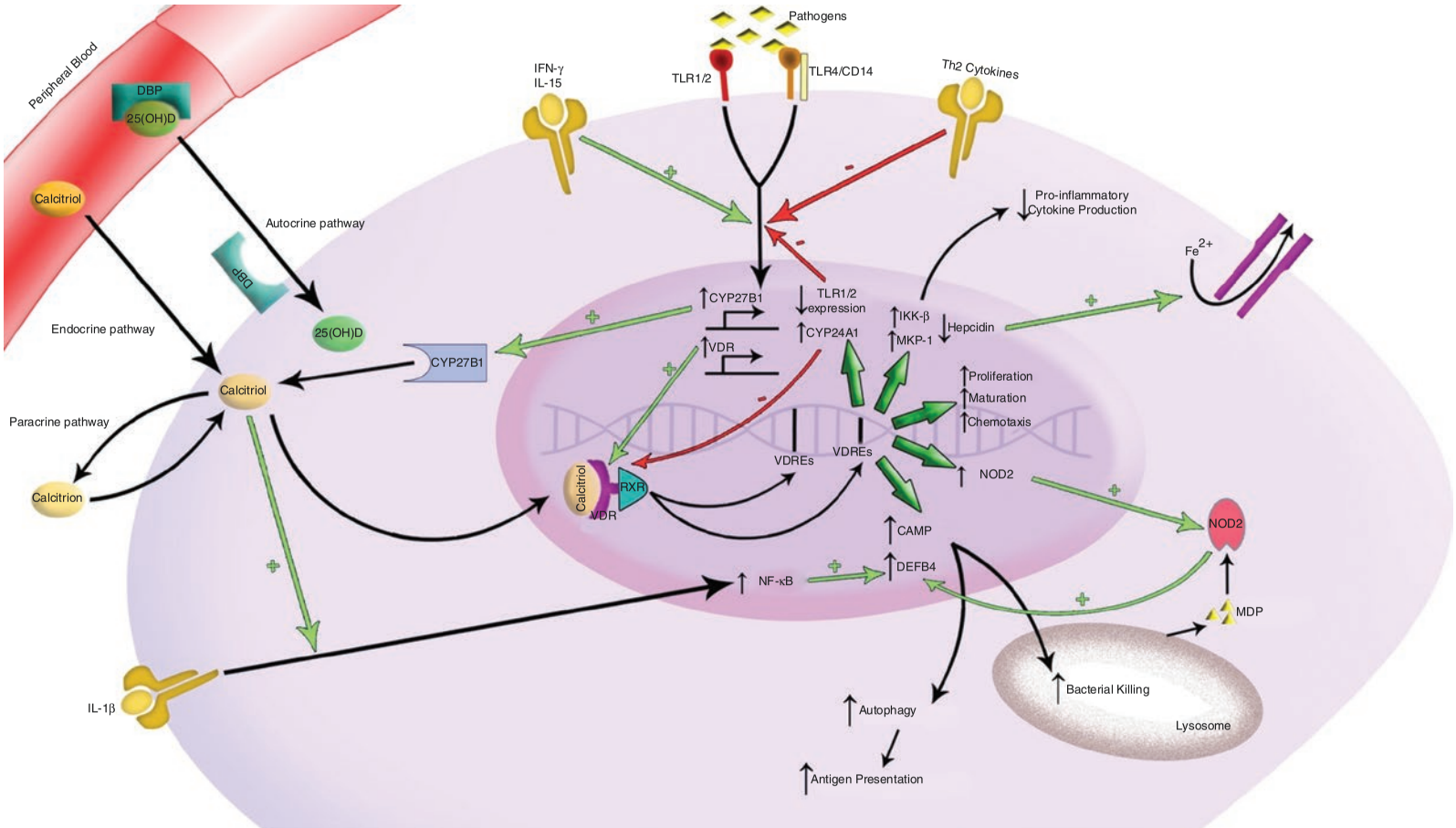

Fig. 2.2 VitD in innate immunity. DBP VitD-binding protein, IL interleukin, IFN interferon, TLR toll-like receptor, Th T-helper, NOD nucleotide-binding oligomerization domain-containing protein 2, MDP muramyl dipeptide, VDR VitD receptor, IKK I kappa B kinase, MKP mitogen-activated protein kinase phosphatase, 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].

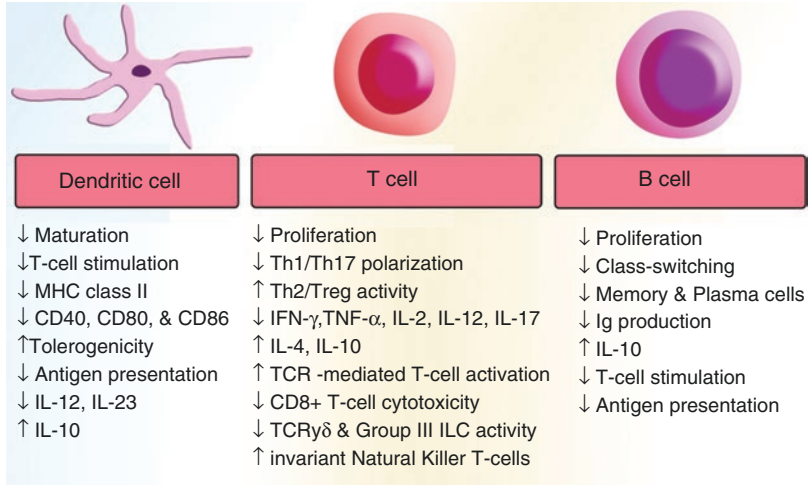

Fig. 2.3 VitD in adaptive immunity. MHC major histocompatibility complex, IL interleukin, Th T-helper, Treg regulatory T cell, IFN interferon, TNF tumor necrosis factor, TCR T-cell receptor, ILC innate lymphoid cell, Ig immunoglobulin

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 $1,25(OH)_2D$-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]. $1,25(OH)_2D$ 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 $1,25(OH)_2D$ [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. $1,25(OH)_2D$ 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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