frontiers in Immunology

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Wendy Dankers[1], Edgar M. Colin[2], Jan Piet Van Hamburg[1], Erik Lubberts[1*]

[1]Rheumatology and Immunology, Erasmus MC, University Medical Center, Netherlands, [2]Rheumatology, ZGT, Netherlands

Submitted to Journal:
Frontiers in Immunology

Specialty Section:
Inflammation

ISSN:
1664-3224

Article type:
Review Article

Received on:
12 Oct 2016

Accepted on:
29 Dec 2016

Provisional PDF published on:
29 Dec 2016

Frontiers website link:
www.frontiersin.org

Citation:
Dankers W, Colin EM, Van_hamburg J and Lubberts E(2016) Vitamin D in Autoimmunity: Molecular Mechanisms and Therapeutic Potential. *Front. Immunol.* 7:697. doi:10.3389/fimmu.2016.00697

Vitamin D in Autoimmunity: Molecular Mechanisms and Therapeutic Potential

Wendy Dankers[1,2], Edgar M. Colin[1,3], Jan Piet van Hamburg[1,2], and Erik Lubberts[1,2*]

Departments of [1]Rheumatology and [2]Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; [3]Department of Rheumatology, ZGT, Almelo, The Netherlands

***Correspondence**
Erik Lubberts
e.lubberts@erasmusmc.nl

Keywords: Vitamin D, autoimmune disease, supplementation, T cells, B cells, dendritic cells, macrophages

Abstract

Over the last three decades it has become clear that the role of vitamin D goes beyond the regulation of calcium homeostasis and bone health. An important extra-skeletal effect of vitamin D is the modulation of the immune system. In the context of autoimmune diseases, this is illustrated by correlations of vitamin D status and genetic polymorphisms in the vitamin D receptor with the incidence and severity of the disease. These correlations warrant investigation into the potential use of vitamin D in the treatment of patients with autoimmune diseases. In recent years several clinical trials have been performed to investigate the therapeutic value of vitamin D in multiple sclerosis, rheumatoid arthritis, Crohn's disease, type I diabetes and systemic lupus erythematosus. Additionally, a second angle of investigation has focused on unraveling the molecular pathways used by vitamin D in order to find new potential therapeutic targets. This review will not only provide an overview of the clinical trials that have been performed, but also discuss the current knowledge about the molecular mechanisms underlying the immunomodulatory effects of vitamin D and how these advances can be used in the treatment of autoimmune diseases.

1 Introduction

Autoimmune diseases, including rheumatoid arthritis (RA), multiple sclerosis (MS) and Crohn's disease, result from an aberrant activation of the immune system whereby the immune response is directed against harmless self-antigens. This results in inflammation, tissue damage and loss of function of the affected organs or joints. With the increasing prevalence of autoimmunity in the Western countries (1), also the societal burden of these diseases increases. Although the treatment of autoimmune diseases has improved due to the development of so-called biologics like tumor necrosis factor alpha (TNFα) inhibitors, a large proportion of patients is still not adequately responding to these treatments (2). Therefore it is still important to improve current therapies or to uncover new treatment options.

In this context, the immunomodulatory effects of vitamin D provide opportunities to enhance the treatment of autoimmune diseases. Firstly, given the high prevalence of vitamin D deficiency in patients suffering from autoimmunity, vitamin D supplementation might decrease disease

severity or augment the therapeutic effect of current medication. Secondly, knowing the molecular mechanisms underlying the immunomodulatory effects could lead to the discovery of new potential therapeutic targets. Therefore, this review will explore the advances that have been made in both clinical trials and molecular studies. In addition, it will give an overview of the challenges that still remain before the immunomodulatory effects of vitamin D can be utilized in clinical practice.

2 Vitamin D metabolism, signaling and function

Vitamin D, or cholecalciferol, is a secosteroid hormone that can be obtained from dietary sources, but that is predominantly synthesized in the skin from 7-dehydroxycholesterol in response to UV light (figure 1). Cholecalciferol is bound by vitamin D binding protein (DBP) and transported to the liver. In the liver various cytochrome p450 (Cyp) vitamin D hydroxylases convert cholecalciferol into $25(OH)D_3$. Cyp2R1 is considered to be the primary 25-hydroxylase responsible for this process. Subsequently DBP transports $25(OH)D_3$ to the kidneys, where the 1α-hydroxylase Cyp27B1 converts $25(OH)D_3$ into $1,25(OH)_2D_3$. $1,25(OH)_2D_3$, also called calcitriol, is the active vitamin D metabolite. To control calcitriol concentrations, the 24-hydroxylase Cyp24A1 hydroxylates $25(OH)D_3$ or $1,25(OH)_2D_3$ at C-24, yielding the less active metabolites $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, respectively (3). The level of $1,25(OH)_2D_3$ is therefore mainly determined by the balance between Cyp27B1 and Cyp24A1. Two proteins that are important for regulating this balance are fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH). FGF23 shifts the balance towards Cyp24A1 and therefore inactivation of vitamin D signaling, and is induced by high concentrations of $1,25(OH)_2D_3$ and low serum phosphate. On the other hand, PTH favors the balance towards Cyp27B1 and activation of vitamin D signaling. PTH is inhibited by high concentrations of $1,25(OH)_2D_3$ and induced by low serum calcium (3) (figure 1).

$1,25(OH)_2D_3$ initiates its signaling cascade by binding to the vitamin D receptor (VDR), which is a nuclear receptor that acts as a transcription factor. VDR binds to vitamin D responsive elements (VDREs) in the DNA, mostly to so-called DR3-type VDREs that are characterized by two hexameric core binding motifs separated by 3 nucleotides. In the absence of ligand, VDR is mostly bound to non-DR3-type VDREs and is associated with co-repressor proteins. When $1,25(OH)_2D_3$ binds to VDR, this induces a conformational change leading to the formation of two new protein interaction surfaces. One is for binding with heterodimeric partners to facilitate specific DNA binding, such as retinoid X receptor (RXR), and the other is for recruitment of co-regulatory complexes that will exert the genomic effects of VDR (4). Furthermore, there is a shift in binding to primarily DR3-type VDREs (5). Interestingly, although RXR has multiple binding partners, specifically with VDR it will bind to the DR3-type elements. This indicates that the heterodimerization of VDR and RXR is important for functioning of the VDR (6). However, research in colorectal cancer cells has shown that 25% of the VDR binding sites are not enriched for RXR (7). No direct data on colocalization of VDR and RXR in immune cells has been reported, although Handel *et al.* found a significant overlap between VDR in $CD4^+$ T cells and RXR in a promyelocytic leukemia cell line (8). Therefore it is currently unknown whether the rate of VDR/RXR colocalization differs between cell types. Also, the functional consequence of VDR binding with or without RXR remains to be understood.

The best known function of $1,25(OH)_2D_3$ is the maintenance of calcium homeostasis by facilitating the absorption of calcium in the intestine. However, in the presence of low

$1,25(OH)_2D_3$ levels, calcium will be mobilized from the bone rather than the intestine. If these conditions are prolonged, this may lead to osteomalacia and rickets, both well-known clinical signs of vitamin D deficiency. An overview of the current knowledge on the role of vitamin D signaling in calcium homeostasis was recently given by Carmeliet *et al*. and will not be discussed here (9). The first hint that vitamin D might also be important for extraskeletal health came from mycobacterial infections like tuberculosis, in which vitamin D was used as a treatment before antibiotics were discovered (10). The discovery that the VDR is expressed in almost all human cells has further increased the attention for the extraskeletal effects of vitamin D. As a result, vitamin D deficiency has now not only been linked to bone health, but also for example cancer, cardiovascular diseases and autoimmune diseases (9).

3 Vitamin D and autoimmune diseases

Since the discovery of the VDR on blood lymphocytes (11, 12), the effects of vitamin D on the immune system and immune-related diseases became the subject of a large number of studies. In this context, it was discovered that supplementation with $1,25(OH)_2D_3$ could prevent both the initiation and progression of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), experimental models of MS and RA, respectively (13-15). In addition, VDR deficiency aggravated arthritis severity in human TNFα transgenic mice (16). Similarly, vitamin D deficiency increased enterocolitis severity in IL-10 knock-out (KO) mice, which are used as a model system for inflammatory bowel diseases (IBD). Treatment with $1,25(OH)_2D_3$ decreased disease symptoms in both the IL-10 KO mice and in the dextran sulfate sodium (DSS)-induced colitis model (17, 18). Finally, treatment with $1,25(OH)_2D_3$ reduced the incidence of diabetes in non-obese diabetic (NOD) mice (19, 20) and the severity of systemic lupus erythematosus in MRL/1 mice (21).

These studies in experimental autoimmune models underscore the need to examine whether there is a protective role for vitamin D in human autoimmune diseases. In the last decades numerous studies have investigated the link between vitamin D and the incidence and severity of autoimmune diseases. One of the first indications was the correlation between increasing MS prevalence and increasing latitude, and consequently with decreasing sunlight exposure. Exceptions to this gradient can at least partially be explained by genetic variants (like the HLA-DRB1 allele) or lifestyle differences, such as high fish consumption (22). The relation between latitude and disease prevalence was also found for other autoimmune diseases such as type I diabetes (T1D) and IBD (23, 24). Further strengthening the link between sun exposure and autoimmunity is the finding that the risk of developing MS is correlated with the month of birth, with for the northern hemisphere a higher risk in April and a lower risk in October and November (25, 26). Importantly, this correlation can only be found in areas where the UV exposure changes during the year (25).

Next to UV exposure, vitamin D can also be obtained from dietary sources and supplements. A meta-analysis by Song *et al.* found that the incidence of RA is inversely correlated with vitamin D intake, both when considering dietary intake and supplements or supplements alone (27). In addition, vitamin D supplementation in early childhood might reduce the risk of developing T1D up to 30% depending on the supplementation frequency (28, 29). Also the effect of maternal vitamin D intake on the risk of T1D in the offspring has been investigated, but due to the limited amount of studies there is currently not sufficient evidence to prove a correlation (29).

Investigating the correlation between vitamin D intake and prevalence of autoimmunity is challenging because the measurements of dietary intake and UV exposure are often based on estimations. Therefore, it might be more useful to analyze the correlation between the serum $25(OH)D_3$ level and autoimmunity. Indeed, in many autoimmune diseases patients have a lower serum $25(OH)D_3$ than healthy controls (30-36). In addition, patients with a lower $25(OH)D_3$ level are implicated to have higher disease activity (32, 35, 37). Although it is not clear whether the lower $25(OH)D_3$ level also increases the risk of autoimmunity, the study by Hiraki *et al.* suggests there is a strong correlation between the risk of developing RA and the $25(OH)D_3$ level between 3 months and 4 years before diagnosis (38). It should be noted that all these studies merely demonstrate correlations, so it is still under debate whether the low $25(OH)D_3$ level is the cause or the result of the autoimmune disease.

Another line of evidence that indicates a role for vitamin D in human autoimmunity is the correlation with polymorphisms in the VDR. There are four well-known VDR polymorphisms that have been extensively studied for their potential role in autoimmunity: ApaI, BsmI, TaqI and FokI. All of these polymorphisms have been associated with the risk of developing an autoimmune disease, although it differs between diseases and polymorphisms whether it is protective or a risk factor. Also, ethnicity plays a role in the correlation between the polymorphisms and autoimmune diseases (39-47).

In summary, autoimmune diseases are correlated with $25(OH)D_3$ serum levels, vitamin D intake, UV exposure and VDR polymorphisms. Furthermore, $1,25(OH)_2D_3$ suppresses disease in experimental autoimmune models. Although these data do not prove a causal relationship between vitamin D and autoimmune diseases, they warrant further investigation into whether at-risk individuals and patients could benefit from vitamin D supplementation.

4 Vitamin D as a therapeutic agent in human autoimmune diseases

Despite the beneficial effects of $1,25(OH)_2D_3$ supplementation in experimental autoimmune models, the application of vitamin D derivatives in clinical practice is currently limited to topical use for the treatment of psoriasis (48). The systemic use of vitamin D in the treatment of other autoimmune diseases is still under investigation. Table 1 gives an overview of the placebo-controlled clinical trials investigating the effect of vitamin D supplementation in autoimmune diseases other than psoriasis. Here we discuss these trials and what this means for the therapeutic potential of vitamin D in each of these autoimmune diseases.

4.1 Multiple Sclerosis (MS)

In the field of MS, several trials have been performed in which cholecalciferol was given to the patients, but the results are contradictory. Beneficial effects of cholecalciferol supplementation that have been reported include decrease in expanded disability status scale (EDSS), decrease in MRI lesions, increased functionality and reduced relapse rates (49, 50). Importantly, cholecalciferol has an added effect when used as a supplement to interferon β (IFNβ) treatment (50). On the other hand, two other trials reported no difference in any of these parameters (51, 52). Vitamin D supplementation might also be important in the pre-MS stage, since cholecalciferol supplementation decreased the conversion rate of optic neuritis to chronic MS (53).

Due to the small sample size (no more than 35 patients per group) of these trials, it is difficult to draw conclusions from these data. Although the effect of cholecalciferol on conversion to chronic effect appears promising, this was only one study with 13 treated patients and 11 placebo controls. Therefore, more research is necessary to determine whether therapy with cholecalciferol is beneficial for MS patients.

4.2 Rheumatoid Arthritis (RA)

Despite the beneficial effect of $1,25(OH)_2D_3$ supplementation on experimental arthritis (15), there are to date only three randomized trials investigating the effect of supplementation on disease activity in rheumatoid arthritis. Although the studies performed by Salesi *et al.* and Dehghan *et al.* suggest a beneficial effect on disease activity and relapse rate respectively, neither results reach statistical significance (54, 55). However, Dehghan *et al.* point out that for every ten patients treated with cholecalciferol, relapse would be prevented in one patient. Considering the costs and safety profile of cholecalciferol supplementation, this might be worth following up. Ergocalciferol, the less potent fungal equivalent of human cholecalciferol, had no effect on disease activity and was associated with worse patient-related health assessments (56). Similarly to studies in MS, the major limitation in the three RA studies is the group size, which limits the power of the analyses. Therefore no definitive conclusion can be drawn yet whether vitamin D can be used as a therapeutic agent in RA.

4.3 Crohn's Disease (CD)

Crohn's disease (CD) is a subtype of the inflammatory bowel diseases and investigated intensively for the effect of vitamin D supplementation. However, the difficulty with this disease is that the intestinal inflammation may lead to decreased absorption of the supplemented vitamin D. Nevertheless, for adult patients cholecalciferol supplementation might reduce the risk of relapses, although the difference does not reach statistical significance ($p = 0.06$) (57). Correspondingly, cholecalciferol prevented further increase of intestinal permeability, which may be an early marker of relapse (58). This is even more pronounced when the patients are stratified based on their serum $25(OH)D_3$ level. Additionally, patients with a serum level above 75 nmol/L have significantly lower serum levels of C-reactive protein (CRP, a marker of inflammation) and a non-significant decrease in disease activity as measured with Crohn's Disease Activity Index (58). These studies used 1,200-2,000 IU cholecalciferol daily in adults, but in children there is no difference in disease activity between supplementing 400 and 2,000 IU daily despite a serum $25(OH)D_3$ level that is 25 nmol/L higher in the latter group (59).
When compared to RA and MS, the results for adult CD are more consistently showing a beneficial effect of cholecalciferol treatment. Since group sizes are again small, more research is required to confirm these data.

4.4 Type I Diabetes Mellitus (T1D)

In contrast to the other autoimmune diseases where cholecalciferol supplementation is investigated, in T1D almost all trials use $1,25(OH)_2D_3$ or an analogue. Both forms appear to delay, but not prevent, the progression of β cell destruction in three studies (60-62). On the other hand, no effect of $1,25(OH)_2D_3$ on T1D was observed in studies performed by Bizzarri *et al.* and

Walter *et al.* (63, 64). This lack of effect could be due to the low level of remaining β cell function at the start of the study, suggesting that the therapeutic window for vitamin D supplementation is in the earliest phases of the disease. The study by Li *et al.* found that the protective effect is only visible when the disease duration was less than one year, supporting this hypothesis (62). In T1D the beneficial effects of $1,25(OH)_2D_3$ may lie more in the prevention of disease onset (28, 29) than in treatment of disease, since the destruction of β cells cannot be reversed.

4.5 Systemic Lupus Erythematosus (SLE)

Vitamin D supplementation in SLE might even be more relevant than in the other autoimmune diseases, since 80% of the patients is sensitive for sunlight and therefore protect themselves against UV exposure (65). Two studies supplementing either 2,000 IU daily or 50,000 IU weekly demonstrate decreasing disease activity score, auto-antibody levels and fatigue (66, 67). Conversely, the type I interferon (IFN) signature was unchanged after 12 weeks of 2,000 or 4,000 IU cholecalciferol in another study (68). Since this study was performed in patients with inactive disease, had a short supplementation period and the signature was based on the expression of only three genes, it remains to be determined whether cholecalciferol supplementation truly does not affect the complete IFN signature in patients with active disease.
SLE is the only autoimmune disease is which a larger study was done, with 158 cholecalciferol-treated patients and 89 placebo controls (66). The promising results in this clinical trial await further confirmation before vitamin D can be used therapeutically in these patients.

5 Immune modulation by vitamin D

In addition to exploring the potential of therapeutic vitamin D supplementation, there has been a great deal of research towards the working mechanisms of $1,25(OH)_2D_3$ in cells of the immune system. Since autoimmune diseases are characterized by an over-active immune response, it seems logical that the beneficial effects of vitamin D on autoimmunity are due to effects on the immune system. Furthermore, virtually all immune cells express the VDR, making them susceptible to $1,25(OH)_2D_3$-mediated modulation (11, 12, 69, 70). Various immune cells, including monocytes, dendritic cells, macrophages, B cells and T cells, also have the capability to convert $25(OH)D_3$ into $1,25(OH)_2D_3$ (71-78). This allows for local regulation of the concentration of $1,25(OH)_2D_3$ at the site of inflammation and illustrates an important role for the cells of the immune system in the systemic effects of vitamin D.
Therefore, insight into how $1,25(OH)_2D_3$ modulates the immune system could uncover new therapeutic targets in autoimmune diseases. Here we discuss the effects of vitamin D on various cell types involved in the immune response, the current knowledge about the underlying mechanisms and what this means for the therapeutic potential of vitamin D in autoimmunity (figure 2).

5.1 Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs), which means that their main function is to take up foreign antigens and present them as peptides to T cells on the human leukocyte antigen (HLA) molecules. DCs are predominantly found in an immature state in peripheral

tissues such as the skin, gut and lungs, where they probe the surroundings for potential pathogens. Upon encountering a foreign antigen, they mature and migrate to the lymphoid tissues to stimulate antigen-specific T cells. Depending on the cytokines secreted by the DC, the T cell will differentiate into an effector cell with appropriate pro- or anti-inflammatory properties. Through these actions APCs are crucial in initiating effective adaptive immune responses against pathogens, but also for maintaining self-tolerance and immune homeostasis.

The important role of DCs in autoimmune pathogenesis is illustrated in experimental autoimmune models, where deletion of specific DC subtypes ameliorates, or even prevents, disease onset (79-82). In addition, APCs, including DCs but also macrophages and B cells, are associated with human autoimmunity through the correlation between specific HLA alleles and the risk of developing an autoimmune disease. For example, HLA-DRB1*15:01 is associated with an increased risk for MS (83), while HLA-DRB1*04:01 confers a greater susceptibility to RA (84).

DCs differentiated *in vitro* from monocytes or bone marrow cells in the presence of $1,25(OH)_2D_3$ will remain in an immature-like tolerogenic state. This is characterized by decreased production of pro-inflammatory factors like IL-12 and TNFα and increased anti-inflammatory IL-10 production. These tolerogenic DCs are less capable of promoting proliferation and cytokine production of pro-inflammatory T cells, while they induce the differentiation of T regulatory (Treg) cells (85-87). Furthermore, they specifically induce apoptosis in autoreactive T cells, while not affecting proliferation of other T cells (88). Of note, $1,25(OH)_2D_3$ can only induce this tolerogenic phenotype in DCs when it is added before their maturation. Once a maturation stimulus like lipopolysaccharide (LPS) is present or when the cells have already matured, the effects of $1,25(OH)_2D_3$ on DCs are minimal (89). Aside from *in vitro* differentiated DCs, $1,25(OH)_2D_3$ also induces a tolerogenic phenotype in dermal DCs, Langerhans cells and plasmacytoid DCs, even though there are subtle differences between the effects on these subsets (90-92).

While the tolerizing effects of $1,25(OH)_2D_3$ on DCs are well described, the underlying mechanisms are less clear. Recently, Ferreira *et al.* suggested that a metabolic switch towards glycolysis and activation of the PI3K-Akt-mTOR pathway are the first steps for the generation of tolerogenic DCs by $1,25(OH)_2D_3$ (93). Also the induction of indoleamine 2,3-dioxygenase (IDO) on DCs has been reported to be essential for the induction of a tolerogenic DC (tDC) phenotype and thereby for the beneficial effect of $1,25(OH)_2D_3$ on EAE (94). Although all tDCs promote regulatory T cells (Tregs), the mechanism by which they do this depends on the type of DC. While tDC derived *in vitro* from bone marrow cells promote Tregs via induction of herpesvirus entry mediator (HVEM), tolerized Langerhans cells use TGFβ for this (91, 95). Dermal DCs induce the differentiation of T regulatory 1 (Tr1) cells, another type of regulatory T cell, via IL-10 (91). So in recent years advances have been made to fully understand how $1,25(OH)_2D_3$ modulates DCs, but the picture is not yet complete.

Despite the incomplete understanding of the molecular mechanism behind the effects of $1,25(OH)_2D_3$ on DCs, tDCs generated with $1,25(OH)_2D_3$ alone or in combination with dexamethasone are considered for therapy in autoimmune diseases (96). Their persistent tolerogenic state and the possibility to pulse them with tissue-specific antigens has made them valuable candidates to treat various diseases, including autoimmune diseases (87, 88, 97). This is illustrated in experimental disease models for T1D, MS and RA, where administered antigen-specific tDCs migrate to inflammatory sites and reduce disease activity upon administration (94,

98-100). Importantly, DCs with an increased activation status from patients with autoimmune diseases can become equally tolerogenic in response to $1,25(OH)_2D_3$ as healthy DCs (101-105). Because they can also be pulsed with auto-antigens and they can be generated under current Good Manufacturing Practice (cGMP) conditions, this opens up the way for the use of autologous tDCs in the treatment of human autoimmune diseases (101, 106). Currently the use of tDCs generated with $1,25(OH)_2D_3$ has not been clinically tested. However, tDCs generated using antisense oligonucleotides or Bay11-7082 were found to be safe upon administration in patients with T1D or RA, respectively (107, 108).
It remains to be determined whether these tDCs also have effects on disease activity and whether tDCs generated using $1,25(OH)_2D_3$ could also be used in this context. Increased understanding on how $1,25(OH)_2D_3$, with or without dexamethasone, modulates the DCs can provide insights in how to further optimize the tolerogenic potential of the DCs.

5.2 Macrophages

Macrophages are known for their supreme phagocytic capacities, but they are also important APCs. In a normal immune response, an infection activates tissue-resident macrophages after which they produce inflammatory mediators and recruit other immune cells to eradicate the pathogen. Macrophages can roughly be divided in two categories: the M1 and M2 macrophages. M1 macrophages produce pro-inflammatory mediators like nitric oxide, TNFα, IL-23, IL-12 and IL-1β, whereby they kill pathogens and promote the polarization of T helper cells to Th1 and Th17 cells to assist in the immune response. On the other hand, M2 macrophages produce the anti-inflammatory cytokine IL-10 and are important in wound repair and restoring tissue homeostasis (109).
The role of macrophages in the pathogenesis in autoimmune diseases is illustrated by an increase in macrophages at inflammatory sites (110-113). In addition, macrophages are hyper-activated and produce more pro-inflammatory cytokines, suggesting a dysregulated balance between M1 and M2 cells (111, 114, 115). As a result of their hyper-inflammatory state, they are essential for the development and activation of β-cell specific cytotoxic T cells which leads to insulitis in NOD mice (116). Interestingly, the suppression of EAE by $1,25(OH)_2D_3$ is preceded by a rapid reduction of macrophages in the CNS. This suggests that macrophages are another important target for vitamin D in the suppression of autoimmunity (117).
Notably, $1,25(OH)_2D_3$ has dual roles in macrophage differentiation and activation. In the early stages of infection, $1,25(OH)_2D_3$ stimulates differentiation of monocytes into macrophages (118). Furthermore, toll-like receptor (TLR) triggering or IFNγ-induced activation activates Cyp27B1 and thereby potentiates the conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ (119, 120). $1,25(OH)_2D_3$ obtained via this pathway is then required for producing cathelicidin and for the antimicrobial activity of human monocytes and macrophages (121, 122). In addition, $1,25(OH)_2D_3$ induces IL-1β, either directly or via upregulation of C/EBPβ or Erk1/2 (123, 124). So initially, $1,25(OH)_2D_3$ is essential for effective pathogen clearance.
The hyper-responsiveness of $VDR^{-/-}$ mice to LPS stimulation indicates that in the later stages of infection, $1,25(OH)_2D_3$ plays a role in the contraction of the immune response (125). The anti-inflammatory effect of $1,25(OH)_2D_3$ on macrophages is characterized by decreased production of pro-inflammatory factors like IL-1β, IL-6, TNFα, RANKL, COX-2 and nitric oxide and increased anti-inflammatory IL-10 (115, 125-128). These changes suggest that $1,25(OH)_2D_3$ promotes the M2 phenotype while inhibiting the M1 phenotype, thereby restoring the balance

between these subsets. Finally, $1,25(OH)_2D_3$-treated macrophages have reduced T cell stimulatory capacity (128).

In recent years some advances were made with unraveling the mechanism behind this anti-inflammatory effect of $1,25(OH)_2D_3$ on macrophages. An important target of $1,25(OH)_2D_3$ is thioesterase superfamily member 4 (THEM4), an inhibitor of the NFκB signaling pathway. THEM4 inhibits the direct binding of NFκB to the COX-2 locus and thereby prevents COX-2 transcription (126). Furthermore, THEM4 inhibits IL-6 and TNFα expression by preventing the signaling cascade in which NFκB induces miR-155 to suppress SOCS (125). Whether this THEM4-dependent pathway also inhibits the other pro-inflammatory mediators is not yet clear (115).

The balancing effect of $1,25(OH)_2D_3$ between the pro- and anti-inflammatory status of macrophages is of particular interest in the treatment of autoimmune diseases. Currently, many inflammatory mediators secreted by M1 macrophages, like IL-1β, COX-2, IL-6 and especially TNFα, are already successful therapeutic targets in various autoimmune diseases. However, since current therapies result in systemic reduction of these mediators, patients may become prone to infections. Therefore it is of interest to understand the mechanism by which $1,25(OH)_2D_3$ balances between pro- and anti-inflammatory actions. This may provide insights in how to suppress the pro-inflammatory cytokines only in case of hyper-activation, without affecting the normal immune response.

5.3 B cells

B cells are mostly known for their crucial role in the immune response via the differentiation towards plasma cells and the production of antibodies. However, they also modulate the immune response via antigen presentation and cytokine secretion. In the context of autoimmunity, B cells play a crucial role by the production of autoreactive antibodies. These auto-antibodies, like anti-nuclear antibodies (ANA) in SLE and anti-citrullinated peptide antibodies (ACPA) in RA, can be found in >95% and 70% of patients, respectively (129, 130).

Interestingly, the VDR binds to the promoter region of genes involved in the immune system in lymphoblastoid B cell lines, suggesting a role for B cells in the effect of vitamin D on autoimmune diseases (131). Here we discuss what is known about the direct effects of $1,25(OH)_2D_3$ on B cell differentiation and the three B cell functions of antibody production, cytokine secretion and antigen presentation.

Before B cells become plasma cells that secrete high-affinity antibodies, they have to go through various stages of differentiation, class-switch recombination and somatic hypermutation (132). Various reports indicate that $1,25(OH)_2D_3$ reduces the proliferation of B cells, induces their apoptosis and inhibits immunoglobulin class switching (133-135). This inhibition of differentiation may involve preventing nuclear translocation of NF-κB p65 and thereby inhibiting the signaling pathway downstream of CD40 costimulation (136). On the other hand, $1,25(OH)_2D_3$ stimulates plasma cell development when added to terminally differentiating B cells. Furthermore, it induces the chemokine receptor CCR10 on these plasma cells, promoting their migration towards mucosal sites of inflammation (137). Therefore, it appears that the effect of $1,25(OH)_2D_3$ depends on the activation and differentiation status of the B cells.

Independent of the effect of $1,25(OH)_2D_3$ on B cell differentiation, there is ample evidence that it decreases the antibody production (133-135, 138, 139). Interestingly, the presence of ANA is

correlated with a lower serum $25(OH)D_3$ level even in healthy people without SLE (140), while cholecalciferol supplementation decreases auto-antibody titers (66, 141).
Next to antibody production, B cells also secrete cytokines to influence the inflammatory milieu. Interestingly, VDR binds directly to the promoter region of IL-10 in B cells, thereby inducing the expression of IL-10 (75). However, in a cohort of healthy controls and relapsing-remitting MS patients there was no correlation between IL-10 producing B cells and serum $25(OH)D_3$ levels (142).
There has been limited research towards the effect of $1,25(OH)_2D_3$ on the APC function of B cells. However one study suggests that B cells primed with $1,25(OH)_2D_3$ have decreased CD86 surface expression. Thereby, these B cells are less potent stimulators of naïve T cell proliferation and cytokine production (143).
Altogether, the effect of $1,25(OH)_2D_3$ on B cells is still not completely clear. Currently it is hypothesized that $1,25(OH)_2D_3$ inhibits the pathogenic function of B cells in autoimmunity by preventing plasma cell differentiation and thereby auto-antibody production, by inducing IL-10 production and by inhibiting the antigen presentation capabilities. However, the limited amount of studies warrants further research to support this hypothesis and what role these effects play in the suppression of autoimmunity by $1,25(OH)_2D_3$.

5.4 T cells

Historically, it was thought DCs were the main target of vitamin D and that effects observed on T cells were mediated via DCs. However, it has now become clear that upon activation various T cell populations express the VDR, including $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T cells and TCRγδ cells (12, 144, 145). This makes the T cell another direct immunological target for $1,25(OH)_2D_3$. The effects of $1,25(OH)_2D_3$ on T cells include modulation of cytokine secretion and differentiation, but VDR is also required for the activation of T cell by propagating TCR signaling (77). Since T cells are proposed to play an important role in the pathogenesis of autoimmunity, we will discuss the effects of $1,25(OH)_2D_3$ on the various T cell populations.

5.4.1 $CD4^+$ T cells

$CD4^+$ T cells are a heterogeneous group of cells, including T-helper 1 (Th1), Th2, Th17 and Treg cells. In the normal immune response, Th1 cells are important for fighting intracellular pathogens, Th2 cells for helminth infections and Th17 cells for extracellular pathogens and fungi. On the other hand, Tregs mediate immunological tolerance against self-antigens and harmless foreign antigens such as food and intestinal microbiota. Furthermore, they control the immune response via various mechanisms, including the secretion of anti-inflammatory mediators such as IL-10 and TGF-β (146). However, in autoimmune diseases T cells mediate an immune response against the body itself, suggesting either hyper-activation of the pro-inflammatory T cells or insufficient control by Treg cells, or both.
The importance of the T cells as a target of $1,25(OH)_2D_3$ in experimental autoimmune diseases is illustrated by Mayne *et al.*, who showed that $1,25(OH)_2D_3$ is not able to suppress EAE when the VDR is absent in T cells (147). For these studies they used the CD4-Cre system, resulting in VDR deficiency in both $CD4^+$ and $CD8^+$ T cells. However, in this disease model $CD4^+$ are likely the prime $1,25(OH)_2D_3$ target cells, since other studies show that in this model $CD8^+$ T cells are dispensable for the effects of $1,25(OH)_2D_3$ (148). Further strengthening the hypothesis that the

suppression of EAE by $1,25(OH)_2D_3$ is driven by modulation of $CD4^+$ T cells, is the finding that $1,25(OH)_2D_3$ prevents $CD4^+$ T helper cell migration into the CNS (149). Finally, VDR binding is enriched near SNPs associated with autoimmune diseases in human $CD4^+$ T cells, suggesting that these cells are also important in the effects of $1,25(OH)_2D_3$ in human autoimmunity (8).
Because the effects of $1,25(OH)_2D_3$ differ between the various $CD4^+$ Th cell subsets (150), we will give an overview of the current knowledge on how these individual subsets are modulated by $1,25(OH)_2D_3$ to suppress the autoimmune response.

5.4.1.1 Th1 and Th2 cells

Classically, $CD4^+$ T cells were subdivided into two classes: Th1 and Th2 cells. Th1 cells are characterized by the expression of IFNγ and T-bet, while Th2 cells produce IL-4, IL-5 and IL-13 and express the transcription factor GATA3. In the context of autoimmunity it was long thought that Th1 cells mediate the disease pathogenesis, since mice lacking the transcription factor T-bet are protected against EAE (151). However, the discovery of Th17 cells, which will be discussed in the next section, and the finding that IFNγ is not required for induction of autoimmunity have led to a debate as to whether Th1 cells are important for autoimmune pathogenesis (152, 153). However, since adoptive transfer of myelin-specific $IFN\gamma^+$ cells induces EAE (154), Th1 cells may still play a role in the disease pathogenesis.
Within Th1 cells, some studies suggest that $1,25(OH)_2D_3$ inhibits IFNγ production when added at the first phases of differentiation (155, 156). On the other hand, another study found no effects on IFNγ (150). This contradiction could be explained by the addition of exogenous IL-2 in the first two studies. Since $1,25(OH)_2D_3$ directly downregulates IL-2, exogenous IL-2 might be required for the inhibition of IFNγ by $1,25(OH)_2D_3$ (157, 158). Although these studies indicate that $1,25(OH)_2D_3$ modulates Th1 cells under certain circumstances, given their relatively small role in autoimmune pathogenesis and the low expression of VDR compared to other $CD4^+$ T cell subsets, it is unlikely that they play an important role in the suppression of autoimmunity by $1,25(OH)_2D_3$ (150, 159).
In contrast to Th1 cells, Th2 cells might be protective in Th17-driven autoimmune diseases even though they are pathogenic in the development of asthma and allergies. Studies in experimental arthritis demonstrate that T cell specific overexpression of GATA3 is protective in autoimmunity due to suppression of Th17 responses (160). Interestingly, IL-4 is required for $1,25(OH)_2D_3$ to inhibit EAE, suggesting an important role for this cytokine in the effect of $1,25(OH)_2D_3$ (161). In the same model, $1,25(OH)_2D_3$ induces GATA3 and its regulator STAT6. The functional relevance of this upregulation is demonstrated in STAT6-KO mice, where $1,25(OH)_2D_3$ is unable to inhibit EAE development (162). Altogether these studies suggest a role for Th2 induction in the immune suppression by $1,25(OH)_2D_3$.
However, the data on the effect of $1,25(OH)_2D_3$ on Th2 cytokines like IL-4 seems contradictory. When naïve $CD4^+$ T cells or the entire $CD4^+$ T cell population are cultured without polarizing cytokines, $1,25(OH)_2D_3$ induces IL-4 and GATA3 (163, 164). Also, in PBMC of treatment-naïve early RA patients, where IL-4 production is diminished, $1,25(OH)_2D_3$ restores the IL-4 levels to the levels of healthy controls (165). However, when naïve $CD4^+$ T cells, effector $CD4^+$ T cells or total $CD4^+$ T cells are cultured in the presence of IL-4 to induce Th2 polarization, cellular IL-4 production is unaffected or even inhibited by $1,25(OH)_2D_3$ (155, 156). Also when patients are supplemented with cholecalciferol, there is no increased IL-4 production by their T cells (141, 166, 167). Combining these data leads to the hypothesis that $1,25(OH)_2D_3$ promotes Th2

differentiation and IL-4 production to assist in suppression of autoimmunity, but only when no sufficient IL-4 is present. The mechanism behind the precise regulation of IL-4 is of interest, not only for treatment of autoimmunity, but also of allergies and asthma where Th2 cytokines play an important pathogenic role.

5.4.1.2 Th17 cells

In most autoimmune diseases, Th17 cells are considered to be important drivers of disease pathogenesis. Th17 cells are characterized by production of cytokines such as IL-17A, IL-17F, TNFα and GM-CSF and the transcription factor RORC2 (RORγt in mice). They can also be distinguished based on the expression of the chemokine receptor CCR6, which directs migration towards the chemokine CCL20. Their differentiation can be driven by TGFβ, IL-6 and IL-1β, but they require IL-23 to become pathogenic Th17 cells (168). In 2003 two hallmark studies showed that IL-23, and not IL-12, is required for the induction of EAE and CIA (169, 170), suggesting an important role for the IL-23/IL-17 immune pathway in the pathogenesis of autoimmune diseases. Indeed, local IL-17A overexpression in mouse knee joints induces an arthritis-like phenotype with inflammation, bone erosions and damaged cartilage (171). In EAE the pathogenic cells appear to be the ex-Th17 cells, which now express IFNγ and T-bet, indicating the importance of Th17 plasticity in autoimmune diseases (172). In human autoimmunity, for example in RA and SLE, levels of Th17 cells are elevated in the peripheral blood and synovial fluid of patients and correlate with disease activity (173-175). Furthermore, specifically the $CCR6^+$ memory Th cells, which include Th17 cells, are potent activators of synovial fibroblasts (173). We have previously shown that this interaction leads to a pro-inflammatory feedback loop with increased production of IL-17A, IL-6, IL-8 and tissue-destructive enzymes. Via this mechanism, Th17 cells may contribute to local joint inflammation in RA (173). Combining the important role of Th17 cells in autoimmunity and the beneficial effect of $1,25(OH)_2D_3$ on autoimmune diseases, it is hypothesized that $1,25(OH)_2D_3$ suppresses autoimmunity at least partially via the inhibition of Th17 activity.

In support of this hypothesis, the effect of $1,25(OH)_2D_3$ on an experimental model for anti-retinal autoimmunity depends on inhibiting Th17 activity (176). Also *in vitro* $1,25(OH)_2D_3$ decreases expression of pro-inflammatory cytokines like IL-17A, IL-17F and IL-22 in $CD4^+$ T cells, $CD4^+$ memory cells or $CD4^+CCR6^+$ memory cells (165, 177-179). Functionally, this decrease in Th17 activity diminishes activation of synovial fibroblasts, thereby inhibiting the pro-inflammatory loop between these cell types (179). Interestingly, $1,25(OH)_2D_3$ also inhibits the secretion of IL-17A and other Th17 cytokines in the presence of Th17 polarizing cytokines (178, 180).

$1,25(OH)_2D_3$ not only inhibits the activity of Th17 cells, but also Th17 differentiation. When naïve $CD4^+$ T cells are differentiated towards the Th17 lineage *in vitro*, the presence of $1,25(OH)_2D_3$ inhibits Th17-related cytokines and transcription factors such as IL-17A, IL-17F, RORC and CCR6 (150, 159, 181). Functionally, MOG-specific Th17 cells differentiated in the presence of $1,25(OH)_2D_3$ are less capable of inducing EAE upon adoptive transfer (178). Aside from the decreased pathogenicity of the cells, this effect may also be due to a decrease in CCR6, the chemokine receptor required for migration to the CNS (182).

Although the inhibitory effect on Th17 activity is well described, the mechanisms behind it are less clear. First of all, Joshi *et al.* showed that the regulation of IL-17A can be mediated via direct binding of the VDR to the IL-17A promoter. VDR-RXR complexes compete with NFAT for the binding sites in the promoter, after which they recruit RUNX1 and HDAC (histone

deacetylase) to inhibit IL-17A gene expression (178). This competition for the NFAT binding site also occurs at the promoter of IL-2, a known primary $1,25(OH)_2D_3$ target gene, suggesting that this may be a general mechanism that also applies to other NFAT-regulated genes (157). Recruitment of HDAC indicates that epigenetic regulation is also important in the inhibition of IL-17A by $1,25(OH)_2D_3$, especially given the relative epigenetic instability of the IL-17A gene locus (183). Aside from this direct regulation of IL-17A, other mechanisms have also been proposed. One study showed that CHOP is crucial for the inhibitory effect of $1,25(OH)_2D_3$, while a second study indicated IRF8 to be important (159, 181). Yet another study indicated that VDR forms a complex with VDR, RXR, HDAC2 and Smad3 to inhibit Smad7 transcription, thereby preventing IL-17A production (184). Of note, TGFβ is the cytokine that induces Smad3 and Erk, leading to this inhibition of IL-17A, but it is also the cytokine responsible for inducing the VDR (180). How these mechanisms relate to each other remains to be investigated.

5.4.1.3 Th17.1 cells

Before the discovery of Th17 cells it was thought that Th1 cells, characterized by expression of IFNγ, T-bet and CXCR3, were the major drivers of the autoimmune response. The finding that IL-23, and not IL-12, was required for experimental autoimmunity, at first completely shifted the viewpoint towards Th17 cells as the pathogenic drivers of autoimmunity. However, lately more and more studies indicate that the subdivision into Th17 and Th1 is not as linear as previously assumed. Upon stimulation by IL-12 or TNFα Th17 cells can become double producers of IL-17A and IFNγ or even shift towards high IFNγ production with little or no IL-17A. Since these latter cells still express CCR6 and RORC, together with T-bet and CXCR3, they are called non-classic Th1 or Th17.1 cells (185). Currently, it is hypothesized that the Th17.1 cells are more pathogenic than Th17 cells in autoimmune diseases, because they are enriched at the sites of inflammation in several diseases (186, 187).
Interestingly, we have shown that in $CCR6^+$ cells, which includes Th17 and Th17.1 cells, $1,25(OH)_2D_3$ reduces the frequency of $IFN\gamma^+$, $IL\text{-}17A^+$ and $IFN\gamma^+IL\text{-}17A^+$ cells (179). This suggests that $1,25(OH)_2D_3$ can inhibit T helper cell pathogenicity in autoimmunity via the inhibition of Th17 and Th17.1 cells. A similar effect was found in the $CD4^+$ T cells of SLE patients supplemented with 10400 IU cholecalciferol for 6 months (188). Other supplementation studies have not addressed the combined or single expression of IFNγ and IL-17A, but the results on total $IL\text{-}17A^+$ or total $IFN\gamma^+$ cells are ambiguous (141, 166, 167).

5.4.1.4 Regulatory T cells

In contrast to the pro-inflammatory T helper subsets mentioned above, regulatory T cells, or Tregs, suppress the immune response. Tregs express FoxP3, the anti-inflammatory cytokines IL-10 and TGFβ, the inhibitory co-receptor CTLA4 and a high level of CD25. They exert immunomodulatory effects on other immune cells such as macrophages, dendritic cells, $CD8^+$ T cells but also other $CD4^+$ T cells, thereby maintaining immune homeostasis. Their essential role in preventing autoimmunity is demonstrated in patients with a mutation in FoxP3. These patients are suffering from the IPEX syndrome, which is characterized by massive autoimmunity (189). In the autoimmune diseases discussed here it is hypothesized that an imbalance between pro-inflammatory T cells, such as Th17 or Th17.1, and regulatory T cells underlies the immune

pathogenesis. $1,25(OH)_2D_3$ may act by restoring this balance and thereby restoring immune homeostasis.

Indeed, $1,25(OH)_2D_3$ induces $FoxP3^+$ Tregs in the spleen, lymph nodes and spinal cord of EAE mice (178, 184). Additionally, without IL-10 or IL-10-mediated signaling, $1,25(OH)_2D_3$ cannot inhibit EAE (190). In *in vitro* cultures of Tregs, either obtained via *in vitro* polarization or sorted from peripheral blood, $1,25(OH)_2D_3$ induces the production of IL-10, but not FoxP3 (164, 191, 192). Polarized Tregs express a higher level of Treg-associated markers such as CTLA4, PD1 and CD25 and their suppressive capacity is enhanced by $1,25(OH)_2D_3$ (192). Also, the suppressive capacity of Tregs is positively correlated with the serum $25(OH)D_3$ level in MS patients (193). However, when sorted Tregs are used, $1,25(OH)_2D_3$ does not further enhance their suppressive capacity (164, 191). This suggests that $1,25(OH)_2D_3$ optimizes Treg function in order to suppress autoimmunity.

Interestingly, $1,25(OH)_2D_3$ also induces IL-10 production when $CD4^+$ cells are cultured under neutral conditions, and even further in the presence of Th17 polarizing cytokines. Furthermore, in these cultures $1,25(OH)_2D_3$ also induces FoxP3 and CTLA4, while enhancing the suppressive capacity of the cells (163, 177, 178, 180, 181, 184, 194). Because $1,25(OH)_2D_3$ inhibits Th17 polarization while inducing IL-10 in these cultures, it was postulated that $1,25(OH)_2D_3$ may inhibit Th17 activity via IL-10 induction. However, IL-10 is dispensable for the inhibition of IL-17A, suggesting that Th17 inhibition and Treg induction are two independent mechanisms of $1,25(OH)_2D_3$ (150).

On a molecular level three mechanisms have been proposed by which $1,25(OH)_2D_3$ can stimulate a Treg-like phenotype even under Th17 polarizing conditions. Firstly, the VDR can bind to three VDREs in the conserved non-coding sequence of the FoxP3 promoter, thereby directly controlling FoxP3 transcription (178, 194). The second mechanism is by reversing the inhibitory effect of Th17 polarizing cytokines on CTLA4, leading to upregulation of CTLA4 (180). Finally, $1,25(OH)_2D_3$ induces the expression of IDO, which increases the number of Tregs (76). The latter finding is interesting, since IDO was also reported to be important for the induction of tDCs (see section 5.1) (94), suggesting it might be a general target of $1,25(OH)_2D_3$ in the immune system.

Although the *in vitro* data demonstrate that $1,25(OH)_2D_3$ induces Treg cells, not all cholecalciferol supplementation studies find an effect on Tregs. Several studies suggest an increase in the proportion or number of Treg cells based on surface marker expression (141, 166, 195) or based on IL-10 production (52, 167). However, another study did not find this induction in Treg cells (61), and Treg suppressive function is unaffected by cholecalciferol supplementation (167).

Overall, in $CD4^+$ T cells $1,25(OH)_2D_3$ inhibits the pro-inflammatory Th cell functions while stimulating Treg activity. These effects are observed under both healthy and pathogenic conditions, such as in patients with autoimmune diseases (191). Therefore, restoring the disturbed balance between effector T cells and Treg cells may underlie the beneficial effects of $1,25(OH)_2D_3$ on autoimmunity.

5.4.2 $CD8^+$ cytotoxic T cells

In addition to $CD4^+$ T cells, cytotoxic $CD8^+$ T cells comprise the second important class within the T cells. These cells contribute to the immune response by inducing apoptosis in abnormal

cells, for example in case of infection or uncontrolled growth in cancer. In addition they modulate other immune cells by secreting cytokines (196). Although the role of $CD8^+$ T cells in autoimmune diseases is not as well characterized as the role of $CD4^+$ T cells, various studies indicate that they play a role in disease pathogenesis. For example, myelin-specific $CD8^+$ T cells induce EAE in mice, with characteristics of human MS that are not conferred by myelin-specific $CD4^+$ T cells (197, 198). Similarly, hsp60-specific $CD8^+$ T cells induce autoimmune intestinal inflammation (199). More recently it was shown that IL-$17A^+CD8^+$ T cells are enriched in the synovial fluid of psoriatic arthritis patients. These cells do not express cytolytic markers, but their levels are positively correlated with markers of disease activity (200). Since $CD8^+$ T cells have a higher expression of VDR than $CD4^+$ T cells (145), $CD8^+$ T cells may also be a target for $1,25(OH)_2D_3$ in the suppression of autoimmunity.

Indeed, adoptive transfer of $VDR^{-/-}$ $CD8^+$ T cells in Rag-deficient mice induces intestinal inflammation. When $VDR^{-/-}$IL-$10^{-/-}$ $CD8^+$ T cells are transferred the intestinal inflammation is even worse and leads to wasting disease (201). The increased proliferation of $VDR^{-/-}$ $CD8^+$ T cells, even in the naive state, suggests that VDR-induced signaling is required for maintaining quiescence of these cells. Thereby $1,25(OH)_2D_3$ prevented hyper-activation of $CD8^+$ T cells and subsequent autoimmune pathology in diseases such as Crohn's disease (201). In addition to maintaining quiescence, $1,25(OH)_2D_3$ also inhibits the secretion of IFNγ and TNFα by activated $CD8^+$ T cells (202). Finally, topical treatment with calcipotriol decreases the frequency of IL-$17A^+CD8^+$ cells in psoriatic lesions, which is interesting in light of the correlations between these cells and disease activity in psoriatic arthritis (200, 203).

Aside from modulating the activity of the classical $CD8^+$ T cells to reduce autoimmunity, $1,25(OH)_2D_3$ is also important in the development of $CD8\alpha\alpha^+$ T cells. $CD8\alpha\alpha^+$ T cells are self-reactive cells that have a regulatory function by maintaining homeostasis in the gut. In $VDR^{-/-}$ mice the number of these cells is reduced, which may explain the susceptibility of these animals to intestinal inflammation (204).

It is important to note that the effect of $1,25(OH)_2D_3$ is not mediated via the $CD8^+$ T cells in every autoimmune disease, since they were dispensable for the attenuation of EAE by $1,25(OH)_2D_3$ (148). However, it seems that in IBD and psoriatic arthritis the $CD8^+$ T cells are target for $1,25(OH)_2D_3$. It will be of great interest to determine what the role of the $CD8^+$ T cells is in the effect of $1,25(OH)_2D_3$ on other autoimmune diseases. This will not only provide insight into the mechanisms behind the effect of vitamin D, but also about the differences in pathogenesis in the various autoimmune diseases.

5.4.3 Unconventional T cells

Next to the traditional $CD4^+$ and $CD8^+$ T cells, there are also cells expressing the TCR but lacking both CD4 and CD8. These so-called unconventional T cells have a less diverse TCR repertoire and they are not restricted to MHC class I or II. The unconventional T cells include mucosal associated invariant T (MAIT) cells, TCRγδ T cells and natural killer T (NKT) cells.

Although MAIT cells have been implicated to be suppressive in autoimmunity, as reviewed by Godfrey *et al.* (205), there is currently no data available on the effect of $1,25(OH)_2D_3$ on these cells.

TCRγδ T cells are rapid responders in the event of an infection with intracellular pathogens, due to their recognition of phospho-antigens. Interestingly, they are pathogenic in autoimmune models like EAE and CIA and they produce a wide range of pro-inflammatory cytokines like IL-

17A, IL-17F, GM-CSF, TNFα and IFNγ (206). There is only one study that investigated the effect of $1,25(OH)_2D_3$ on the pro-inflammatory activity of these cells. They demonstrated that TCRγδ T cells express the VDR upon activation. In response to $1,25(OH)_2D_3$ the production of IFNγ and the proliferation of these cells was inhibited (144). Currently it is thought that the main pathogenic action of the TCRγδ T cells in autoimmunity is the secretion of IL-17A (206). Unfortunately, there is no data available yet that describes the effect of $1,25(OH)_2D_3$ on this cytokine, or any of the other cytokines secreted by the TCRγδ T cells.

The last subset of unconventional T cells that will be discussed here are the NKT cells. They recognize glycolipid antigens and are thereby involved in the protection against a wide range of pathogens. Upon TCR stimulation, NKT cells can rapidly secrete various pro-inflammatory cytokines, including IL-4, IFNγ and IL-17A. NKT cells can be divided into type I and type II NKT cells. Type I NKT cells are also called invariant NKT (iNKT) cells due to their invariant TCR. Type II NKT cells have a variable TCR and are therefore called the variant NKT cells. The exact role of NKT cells in the pathogenesis of autoimmune disease is not yet completely clear. They are pathogenic in CIA, but they are protective in EAE, T1D and SLE (161, 207).

Interestingly, VDR is required in the thymus for the development of functionally mature iNKT cells. Furthermore, the iNKT cells in $VDR^{-/-}$ mice are hyporesponsive to TCR stimulation (208). In addition, the protective effect of $1,25(OH)_2D_3$ in EAE is partially dependent on iNKT cells, possibly via inducing IL-4 in these cells (161). These data suggest that $1,25(OH)_2D_3$ promotes a suppressive function of iNKT cells. However, given the two-sided effect of iNKT cells in the different autoimmune diseases, further research is needed to fully examine the effect of $1,25(OH)_2D_3$ on iNKT cell activity and what this means for each individual disease.

5.5 Innate lymphoid cells

Recently a new group of cells became the center of attention in the field of immunology; the innate lymphoid cells (ILC). ILCs play an important role in tissue repair, tissue homeostasis and the immune response against bacteria, viruses and fungi. ILCs can be grouped into three classes; (i) the group 1 ILCs (ILC1) that secrete IFNγ and depend on T-bet expression, (ii) the group 2 ILCs (ILC2) that secrete type 2 cytokines such as IL-5 and IL-13 and depend on GATA3 and (iii) the group 3 ILCs (ILC3) that secrete IL-17A and/or IL-22 and depend on RORC (209) .

The ILC1s include natural killer cells, which have been known for a longer time and play a role in the clearance of viruses. Since viral triggers are thought to play a role in the initiation of some autoimmune diseases, the NK cells have been investigated for their role in this context. However, under some circumstances NK cells are protective, while in others they can be pathogenic as recently reviewed by Poggi and Zocchi (210). Also the data on the effect of $1,25(OH)_2D_3$ on NK cells are somewhat contradictory. In an NK cell line, $1,25(OH)_2D_3$ induces the cytolytic killing capacity of NK cells (211), but this effect has not been found in healthy control peripheral blood (212, 213). However, when $1,25(OH)_2D_3$ is added during the *in vitro* differentiation of NK cells from hematopoietic stem cells, the development of NK cells is impaired and their cytotoxicity and IFNγ production are reduced (212). Interestingly, $1,25(OH)_2D_3$ specifically inhibits activation, cytotoxic capacity and pro-inflammatory cytokine production in over-activated NK cells in women with recurrent pregnancy losses (213). This supports a hypothesis in which $1,25(OH)_2D_3$ is not a general inhibitor of the immune response, but rather a regulator of immune homeostasis. Therefore it is of interest whether this abnormal NK activation is also seen in autoimmune diseases and can be modulated by $1,25(OH)_2D_3$.

Based on their cytokine signature, it can be hypothesized that in the context of autoimmunity ILC3 cells play a role in disease pathogenesis. Indeed, an increase in ILC3 cells has been demonstrated in the lesional skin of psoriasis patients (214, 215), in the inflamed intestine of Crohn's disease patients (216), in the peripheral blood of MS patients (217) and in the gut, peripheral blood, bone marrow and synovial fluid of patients with ankylosing spondylitis (218). Furthermore, ILC3 were shown to be responsible for experimental innate-induced colitis (219). Interestingly, in VDR-KO mice, which are susceptible for colitis, the levels of ILC1 and ILC3 are increased (220). On the other hand, calcipotriol treatment did not affect the frequencies of ILC subsets in psoriatic skin lesions after two weeks (203).
Since the research into ILC has only started to expand in recent years, the effects of $1,25(OH)_2D_3$ on these cells have not been investigated extensively. Current data suggests that $1,25(OH)_2D_3$ may also have anti-inflammatory effects on these cells, but more studies are required to distinguish the effects on the different subsets and its role in the protective effect of vitamin D in autoimmunity.

5.6 Indirect immunomodulatory effects

In the previous sections we discussed the direct modulatory effects of $1,25(OH)_2D_3$ on various cells of the immune system. However, $1,25(OH)_2D_3$ and the VDR also affect tissue resident cells, such as hepatic and pancreatic stellate cells, and the inflammatory mediators that they secrete (221, 222). This indirect mechanism of immune modulation by $1,25(OH)_2D_3$ is also relevant in autoimmune diseases. For example, in RA the interaction between T cells and synovial fibroblasts contributes to disease pathogenesis (173). Therefore it is also of interest to study the effect of $1,25(OH)_2D_3$ on the tissue-resident cells in the context of autoimmunity.
Similar to the tissue-resident tissue cells in liver and pancreas, $1,25(OH)_2D_3$ also directly affects RA synovial fibroblasts. Not only is the IL-1β-induced production of tissue-degrading matrix metalloprotease 1 (MMP1) inhibited, also the infiltration capacity of RA fibroblasts is reduced upon treatment with $1,25(OH)_2D_3$ (223). But this effect on tissue-resident cells is not only found in the synovial cells. It was also shown that the VDR is required for intestinal homeostasis by limiting the production of IL-6 by epithelial cells through inhibition of the NFκB pathway (224). Finally, $1,25(OH)_2D_3$ also affects brain pericytes, which may be relevant for MS. The pericytes line the epithelial cells of blood vessels and in the brain they are important for maintaining the blood-brain-barrier and neuron functioning. Brain pericytes cells produce less pro-inflammatory genes when exposed to $1,25(OH)_2D_3$ while upregulating anti-inflammatory genes. Interestingly, brain pericytes express Cyp27B1 upon stimulation with TNFα and IFNγ. This indicates that an inflammatory environment promotes the conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$, which then can dampen the inflammation by modulating the pericytes (225).
Overall, the indirect effects of vitamin D and the VDR on immune cells via tissue-resident cells have been underexposed in the past years. However, if we truly want to understand the molecular mechanisms by which $1,25(OH)_2D_3$ acts in autoimmune diseases, these effects are very important for future studies.

6 Future directions

In this review we have discussed the advancements that have been made regarding the clinical effects of vitamin D and the molecular mechanisms that underlie these effects. However, there is still a lot that is unclear at the moment which will be subject of investigation in the coming years.

6.1 Vitamin D supplementation

Based on the current data on the effect of vitamin D supplementation it is still not possible to draw conclusions about the added value for the treatment of autoimmunity. This is due to the low number of trials, small patient numbers and heterogeneity in trial setup. In order to determine the therapeutic value of vitamin D supplementation, there are two big open questions that need to be addressed.

Firstly it is important to assess what serum $25(OH)D_3$ level is required for a beneficial effect of vitamin D in autoimmune diseases. Based on the requirements for calcium homeostasis, current guidelines indicate that a level below 50 nmol/L corresponds with deficiency, between 50 and 74 nmol/L as insufficiency and above 75 nmol/L as a sufficient $25(OH)D_3$ level (226, 227). However, in the context of autoimmunity it is not known whether it is enough to correct deficiency or whether we should strive for an even higher serum $25(OH)D_3$ level. Using 75 nmol/L as a cut-off point, Raftery *et al.* showed that CD patients with sufficient serum $25(OH)D_3$ have significantly higher quality of life and less severe disease as measured by intestinal permeability, LL-37 expression and CDAI (58). Furthermore, in healthy individuals the serum $25(OH)D_3$ level is correlated with number of VDR binding sites in $CD4^+$ T cells. When they have a level above 75 nmol/L, the VDR binding is enriched near genes associated with autoimmune diseases and regulatory T cells (8). However, clinical trials, either with or without placebo controls, do not consistently find immune modulation regardless of the baseline and endpoint serum $25(OH)D_3$ level (table 2). It should be noted that these measurements have been done in the peripheral blood or in cells from the peripheral blood, which is not the site of inflammation and therefore may not be the most relevant place to look for immunological effects.

The second question that is still matter of debate is in what form and dosage vitamin D should be supplemented. In the experimental autoimmune models animals are mostly supplemented with a high dose of $1,25(OH)_2D_3$, but in humans this strategy may lead to hypercalcemia. Therefore most clinical trials use cholecalciferol as the form of choice, although some use $1,25(OH)_2D_3$ or less calcemic analogues like alfacalcidiol. Of note, a study comparing the effects of alfacalcidiol (analogue for $1,25(OH)_2D_3$) with colecalciferol (analogue for cholecalciferol) indicates that in the short term alfacalcidiol might be more effective, but this effect disappears after 12 months (228). Analogues like calcipotriol that are used in the topical treatment of psoriasis have not been tested in the other autoimmune diseases that were discussed here. Other analogues have been developed, which show equal or better immunomodulatory potential and have been successfully used in experimental autoimmune diseases (191, 229-233). The only analogue that was used in clinical trials was alfacalcidiol, mainly in type 1 diabetes patients (table 1). However, the effects of alfacalcidiol do not seem better than calcitriol, and at the same dosage there were no severe side effects from either alfacalcidiol or calcitriol (60, 63, 64). More research into the actual effects of vitamin D analogues on human autoimmune disease is required for establishing whether these analogues can be used safely and effectively. Furthermore, in the clinical trials performed so far there were no serious adverse events after cholecalciferol supplementation. Therefore it is important to establish the added value of the vitamin D analogues compared to cholecalciferol supplementation. Currently, cholecalciferol is the most used supplementation form in clinical practice. Vitamin D supplementation guidelines indicate a maximum safe dose of 4,000 IU cholecalciferol per day for healthy adults (226). However, no adverse effects were

found with dosages of up to 50,000 IU cholecalciferol weekly for 12 weeks, or 100,000 IU weekly for 1 month followed by 100,000 IU monthly for 5 months (54, 141, 167). Interestingly, the dose-escalation regime used by Burton *et al.* and 20,000 IU weekly by Smolders *et al.* did not elicit hypercalcemia despite reaching a serum $25(OH)D_3$ level of 400 and 380 nmol/L, respectively (49, 167).

In considering the best strategy for cholecalciferol supplementation it should also not be forgotten that $1,25(OH)_2D_3$ may have a synergistic effect with other treatments. For example, *in vitro* studies have shown that $1,25(OH)_2D_3$ synergizes with retinoic acid (an active vitamin A metabolite) or dexamethason in the inhibition of Th17 pathogenicity (165, 234). Also in monocytes the combination of dexamethasone and $1,25(OH)_2D_3$ has added effects over the compounds separately, partially because $1,25(OH)_2D_3$ enhances the effects of the glucocorticoid receptor (235, 236). Furthermore, we have previously shown that $1,25(OH)_2D_3$ has an added effect on TNFα blockade in inhibiting the pro-inflammatory loop between Th17 cells and RASF in RA, suggesting that vitamin D combined with anti-TNFα could yield a better treatment response in the treatment of RA patients (179). Finally, combining $1,25(OH)_2D_3$ with Lovastatin has an added therapeutic effect on EAE. This is due to the inhibition of RhoA-ROCK signaling in autoreactive T cells, leading to decreased expression of Cyp24A1 and thereby less inactivation of $1,25(OH)_2D_3$ (237). Altogether, these data indicate that it may be worthwhile to investigate the addition of cholecalciferol to current treatments like anti-TNFα, or to combine cholecalciferol with for example retinoic acid or statins. Due to the synergy between $1,25(OH)_2D_3$ and these already approved drugs, a lower dose of cholecalciferol may be sufficient for achieving beneficial clinical effects.

Currently several clinical trials are ongoing and recruiting patients in MS (clinicaltrials.gov identifier NCT01490502), RA (NCT02243800) and IBD (NCT02704624, NCT01046773, NCT02208310) for which the results are expected in the coming 3 to 5 years. Hopefully they can provide more insight into the answers on these remaining questions. However, to firmly establish the added value of cholecalciferol supplementation, large multi-center trials are required. Ideally, in these trials the patients should be randomized into different treat-to-target arms, in which every arm has a target $25(OH)D_3$ serum level, such as 75, 100 and 150 nmol/L. Since the effect of cholecalciferol alone is probably not sufficient to control disease activity, patients should receive standard care following pre-defined, harmonized treatment protocols in addition to the cholecalciferol supplementation.

6.2 Molecular mechanisms underlying immunomodulation

In addition to the studies where cholecalciferol has been supplemented, attention has also focused on understanding the immunomodulatory effects of $1,25(OH)_2D_3$ on a cellular level. Based on the current knowledge, $1,25(OH)_2D_3$ reduced the pathogenicity of dendritic cells, macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells. Similar effects have been observed in γδ T cells, iNKT cells and ILCs, but more research is necessary to confirm these data (see section 5). It should be noted that $1,25(OH)_2D_3$ does not merely work as an anti-inflammatory agent. Instead, $1,25(OH)_2D_3$ assists in maintaining the balance between a pro- and anti-inflammatory state and is thereby able to restore the disturbed balance that is associated with autoimmunity.

This balancing effect of $1,25(OH)_2D_3$ is best illustrated in monocytes and macrophages, where it has pro-inflammatory effects in the early stages of activation but later shifts to an anti-inflammatory state (238). Therefore it is interesting to study the effects of $1,25(OH)_2D_3$ in more

detail in the various stages of differentiation and activation from monocyte to macrophage. The Carlberg lab has performed ChIP-seq experiments in the monocytic THP-1 cell line at early time points (5). Detailed studies have revealed several primary target genes such as ASAP2 and THBD (239-241), but also identified Bcl6 as a primary target that mediates important secondary responses (242). Next to the primary target genes, combining the ChIP-seq dataset with publically available ChIA-PET and FAIRE-seq datasets has improved the knowledge on VDR binding kinetics (243, 244).

This is just an example of how next generation sequencing techniques can be combined to yield more understanding of the molecular mechanisms behind the effects of $1,25(OH)_2D_3$. Since it has already been shown that $1,25(OH)_2D_3$ has different effects on every cell type, even closely related cell types such as Th1 and Th17 (150), it will be interesting to study VDR DNA binding and identify primary target genes in separate cell types. This will give insight into the similarities and differences between the effects of $1,25(OH)_2D_3$ on each cell, and what will be important to balance the immune response in patients with autoimmune diseases.

7 Conclusion

Although various studies have shown a beneficial effect of cholecalciferol supplementation in autoimmune diseases, there are also studies that do not find any effect on disease parameters. This might be due to the supplementation strategy or the subjects included in the study, which are issues that should be addressed in properly designed multi-center clinical trials.

However, it is also possible that systemic cholecalciferol supplementation is not sufficient to establish effects in every patient. Therefore, another way to use the immunomodulatory effects of vitamin D to the advantage of patients with autoimmune diseases, is to mimic the effects by targeting important pathways within immune cells. In order to do this, it is crucial to understand the working mechanisms of $1,25(OH)_2D_3$. In the coming years attention should be paid towards unraveling these molecular mechanisms to optimize the therapeutic potential of vitamin D.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Author contributions

WD has performed literature research, designed the review layout and written the review. EC has designed the review layout, contributed to the clinical section and revised the manuscript. JH has designed the review layout and revised the manuscript. EL has designed the review layout, contributed to the molecular section and revised the manuscript.

Funding

This work is supported by a grant provided by the Dutch Arthritis Association.

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Table 1| Overview of randomized controlled trials with vitamin D supplementation in autoimmune diseases. ASA 5-aminosalicylzuur (sulfasalazine); CDAI Crohn's disease activity index; CQ Chloroquine; CRP C-reactive protein; ECLAM European consensus lupus activity measurement; EDSS Expanded disability status scale; ESR Erythrocyte sedimentation rate; FCP Fasting c-peptide; Gd Gadolinium; HAQ Health assessment questionnaire; HCQ Hydroxychloroquine; IU International Units; LADA Latent autoimmune diabetes in adults; MTX Methotrexate; PCP C-peptide after 75g glucose; QoL Quality of life; RCT Randomized controlled trial; RRMS Relapsing-remitting multiple sclerosis; SLEDAI Systemic lupus erythematosus disease activity index; DAS28 Disease activity score for 28 joints; VAS Visual analogue scale.

Trial	Disease	Trial design	Inclusion criteria	Groups	Supplementation dosage	Supplemental calcium	Other medication	Baseline $25(OH)D_3$ in treated group (nmol/L)	Endpoint $25(OH)D_3$ in treated group (nmol/L)	Main clinical findings
Burton 2010 (49)	MS	Open-label RCT, 52 weeks.	MS without a relapse within 60 days. EDSS 0-6.5. Serum $25(OH)D_3$ < 150 nmol/L.	N=25 cholecalciferol, N=24 placebo	Dose escalation: up to 280.000 IU per week in 23 weeks, stay 6 weeks, then reduce to 0 in 20 weeks, then 3 weeks without	1200 mg daily	Continuation of MS medication, placebo-treated patients could take up to 4000IU cholecalciferol and supplemental calcium if desired. In case of relapse patients received steroids as judged by the treating physician	80	Up to 400 nmol/L after the peak of dosage, 200 nmol/L at the end of the trial	Lower proportion of patients with an increase in EDSS at the end of the trial. Trend towards reduced relapse rate.
Mosayebi 2011 (52)	MS	Double-blind RCT, 6 months (October-March).	MS with a relapse in the last year. More than 3 lesions on MRI. EDSS 0-3.5.	N=28 cholecalciferol, N=34 placebo	300.000 IU monthly (intramuscular)	No	IFNB-1a	25	150	No effect on EDSS. No effect on Gd-enhancing lesions.
Soilu-Hänninen 2012 (50)	MS	Double-blind RCT, 12 months.	RRMS with at least 1 month IFNB-1b treatment. Serum $25(OH)D_3$ < 85nmol/L.	N=34 cholecalciferol, N=32 placebo	20.000 IU weekly	No	IFNB-1b	54	110	Reduced number of Gd-enhancing lesions, but no effect on other MRI parameters. Trend towards reduced EDSS.
Kampman 2012 (51)	MS	Double-blind RCT, 96 weeks.	MS with an EDSS<4.5.	N=35 cholecalciferol, N=33 placebo	20.000 IU weekly	500 mg daily	46% of patients in both groups were treated with IFNβ, 3% with glatiramer acetate and 3% in the placebo group with natalizumab	55	123	No effects on EDSS, relapse rate, function or fatigue.
Derakhshandi 2013 (53)	MS	Double-blind pilot RCT, 12 months.	Optic neuritis patients without MS.	N=13 cholecalciferol, N=11 placebo	50.000 IU weekly, when reaching serum $25(OH)D_3$ of 250 nmol/L switch to a maintenance dose	No	3x 1g methylprednisolone per day i.v., then oral prednisolon	38	Unknown	Decreased incidence-rate ratio of demyelinating plaques. Reduced risk of progression to MS.
Salesi 2012 (54)	RA	Double-blind RCT, 12 weeks.	RA with DAS28>3.2. At least 24 weeks MTX treatment.	N=50 $25(OH)D_3$, N=48 placebo	50.000 IU weekly	No	MTX Prednison, HCQ and CQ were allowed	107	125	Modest, non-significant, improvement in tender joint count, swollen joint count, ESR and VAS.
Dehghan 2014 (55)	RA	Double-blind RCT, 6 months.	RA in remission for at least 2 months. Serum $25(OH)D_3$ <75 nmol/L	N=40 cholecalciferol, N=40 placebo	50.000 IU weekly	No	Prednison, MTX and HCQ allowed	<75	Unknown	Non-significant decrease in relapse rate.
Hansen 2014 (56)	RA	Double-blind RCT 12 months.	RA. Serum $25(OH)D_3$ between 15,25 and 62,25 nmol/L	N=11 cholecalciferol, N=11 placebo	4 weeks: 50.000 IU 3x weekly; 11 months: 50.000 IU 2x monthly; when serum was below 62,5 nmol/L: 50.000IU weekly for 8 weeks	500 mg 3x daily	SPF65	63	75 (after two months)	No effects on DAS28, HAQ or physician global assessment of RA. Non-significant increase in pain. Increased patient assessment of global health and patient global assessment of RA.

Jørgensen 2010 (57)	CD	Double-blind RCT, 1 year.	Crohn's disease in remission (CDAI<150) for at least 4 weeks.	N=46 cholecalciferol, N=48 placebo	1200 IU daily	1200 mg daily	Azothioprine (39-44% of participants)	70	95	Trend towards reduced relapse (hazard ratio of 0.44)
Wingate 2014 (59)	CD	Double-blind RCT, 6 months.	Children with quiescent Crohn's disease	N=35 2000 IU cholecalciferol, N=34 400 IU cholecalciferol	400 IU or 2000 IU daily depending on randomization	No	Multivitamins (without vitamin D). Normal IBD medication (36% 5-ASA, 57% immunomodulator, 30% biologics)	63	70 (400IU) or 86 (2000IU)	No difference between the groups in CDAI, ESR or CRP.
Raftery 2015 (58)	CD	Double-blind RCT, 3 months.	Adults with CD in remision (CDAI<150) and stable therapy for 3 months.	N=13 cholecalciferol, N=14 placebo	2000 IU daily	Only when already on it for bone health	Normal IBD medication (51% 5-ASA, 67% immunomodulator, 7% anti-TNFα).	70	90	Intestinal permeability was stable in the treated group, but increased in the placebo group. Reduced CRP, increased QoL and trend towards decreased CDAI in patients with serum $25(OH)D_3$ > 75 nmol/L.
Li 2009 (62)	T1D	Prospective RCT, 12 months.	LADA patients with diagnosis < 5 years	N=17 alfacalcidol, N=18 unsupplemented	0,25 µg twice daily	No	Insulin therapy in both groups	63	Unknown	Stable FCP while decline in control group, same trend for PCP. Especially pronounced when disease duration < 1 year.
Bizzarri 2010 (64)	T1D	Double-blind RCT, 24 months.	Recent-onset T1D	N=15 calcitriol, N=12 placebo	0,25 µg daily	No	Insulin therapy in both groups	<50	+ 3.9%	After 12 months the decline is FCP is slower in treated group, but not anymore after 24 months.
Walter 2010 (63)	T1D	Double-blind RCT, 18 months.	Adults with recent-onset T1D	N=20 calcitriol, N=18 placebo	0,25 µg daily	No	Insulin therapy in both groups	25 pg/ml (1,25OHD3)	30 pg/ml (1,25OHD3)	No changes in C-peptide or insulin dose.
Gabbay 2012 (61)	T1D	Double-blind RCT, 18 months.	Patients with recent onset T1D (age > 7). PCP > 0,06 ng/mL.	N=17 cholecalciferol, N=19 placebo	2000 IU daily	No	Insulin therapy in both groups	65	150	Decreased progression to undetectable C-peptide. Enhanced stimulated C-peptide after 12 months. Decreased decay of stimulated C-peptide after 18 months.
Ataie-Jafari 2013 (60)	T1D	Single-blind RCT, 6 months.	Patients with recent onset T1D	N=29 alfacalcidol, N=25 placebo	0,25 µg once daily, or twice if blood calcium levels allowed it	No	Insulin therapy in both groups	32.5	Unknown	Better preservation of C-peptide and lower insulin dose. Stronger effect in males than in females.
Abou-Raya 2013 (66)	SLE	Double-blind RCT, 12 months.	SLE with SLEDAI>1. Serum $25(OH)D_3$ <75 nmol/L.	N=158 cholecalciferol, N=89 placebo	2000 IU daily	Yes, unknown dose	6% corticosteroids, 80% antimalarials, 26% AZA, 27% ACE inhibitors/ARB	50	98	Decrease in SLEDAI and ESR.
Lima 2014 (67)	SLE	Double-blind RCT, 24 weeks.	Juvenile onset SLE SLEDAI<12	N=20 cholecalciferol, N=20 placebo	50.000 IU weekly	No	Unknown, but stable during trial	50	78	Decrease in SLEDAI, trend to decrease in ECLAM and decrease of fatigue related to social life.
Aranow 2015 (68)	SLE	Double-blind RCT, 12 weeks.	Adult SLE with IFNa signature. Stable inactive disease. Anti-dsDNA positive. Serum $25(OH)D_3$ <50 nmol/L.	N=18 4000 IU cholecalciferol, N=17 2000 IU cholecalciferol N=19 placebo	2000 IU or 4000 IU daily	No	Unknown	28	75	No difference in IFN signature (based on 3 genes) or disease activity.

Table 2| Overview of clinical trials looking at immunological parameters after vitamin D supplementation. aTreg Activated memory regulatory T cells; BAFF B-cell activating factor; CM Central memory; CS Class-switched memory; DN Double negative; EM Effector memory; iTreg Induced regulatory T cells; IU International Units; moDC Monocyte-derived dendritic cell; MZ Marginal zone; rTreg Resting regulatory T cells; TE Terminal effector; tTreg Thymic regulatory T cells; # number; [ ] concentration.

Trial	Disease	Supplementation strategy	Mean baseline 25(OH)D_3	Mean endpoint 25(OH)D_3	PBMC	T cells		B cells	Innate immune cells (DC, NK)	Cytokines and antibodies in serum or plasma
						$CD4^+$	**$CD8^+$**			
Bock 2011 (195)	Healthy	3 months 140.000 IU cholecalciferol monthly or placebo	64±29 nmol/l	~138 nmol/l		Increased % of Tregs				
Smolders 2010 (167), Knippenberg 2011 (142), Peelen 2013 (158)	MS	12 weeks 20.000 IU cholecalciferol daily (no placebo group)	50 (31-175) nmol/l	308 (151-535) nmol/l		No difference in % or function of Tregs, either naive or memory. Increased production of IL-10 and decreased IL-17A/IL-4 ratio in T cells from PBMC cultures.	No relation between % $IL\text{-}10^+$ or $IL\text{-}17^+$ $CD8^+$ and serum 25(OH)D_3. No change in % $IL\text{-}10^+$ or $IL\text{-}17^+$ $CD8^+$.	No difference in %, # or differentiation status of circulating B cells.		No difference in BAFF. No change in immuno-globulins.
Kimball 2011 (245)	MS	Dose escalation: up to 280.000 IU per week in 23 weeks, stay 6 weeks, then reduce to 0 in 20 weeks, then 3 weeks without (trial: Burton *et al*, 2010)	78±27 nmol/l	179±76 nmol/l	Decreased PBMC proliferation in response to certain MS-associated antigens					
Mosayebi 2011 (52)	MS	6 months 300.000 IU cholecalciferol or placebo i.m. monthly	~25 nmol/l	~140nmol/l	Decreased PBMC proliferation upon PHA stimulation. No difference in IFNγ, but increase in IL-10 and TGFβ production in these cultures.					
Sotirchos 2016 (188)	MS	6 months 10400 or 800 IU cholecalciferol daily	10400: 68±22 nmol/l 800: 70±21 nmol/l	10400: + 87 (63-112) nmol/l compared to baseline 800: +17 (3-34) nmol/l compared to baseline		High dose, but not low dose, decreases % $IL\text{-}17^+$, but not % $IFN\gamma^+$ or % $IFN\gamma^+IL\text{-}17^+$. High dose, but not low dose, decreases % of EM and $CD161^+$, while decreasing % of CM and naïve. % $IL17^+$ is correlated with % EM For every 12.5 nmol/l increase in serum 25(OH)D_3, the % $IL\text{-}17^+$ $CD4^+$ decreases by 1% (when serum 25(OH)D_3 increases more than 45 nmol/l)	High dose, but not low dose, decreases $CD85j^+$			
Bendix-Struve 2010 (246), Bartels 2014 (103)	CD	1 year placebo vs 1200 IU cholecalciferol daily (trial Jorgensen *et al.* 2010)	33 (16-66) nmol/l	118 (62-154) nmol/l		Over time decrease of IL-6 production is prevented upon supplementation. Increased $CD4^+$ proliferation which is inversely correlated with the IL-10 production.			MoDCs have decreased IL-10, IL-6, IL-8 and IL-1β, CD80 and HLA-DR. The allogeneic stimulatory capacities of moDCs are unaffected.	

Yang 2013 (247)	CD	24 weeks, start with 1000IU cholecalciferol daily, increase to 5000IU daily or until serum 25(OH)D_3 is 100 nmol/L (no placebo group)	40±25 nmol/L	113±48 nmol/L						No change in IL-17, TNFα or IL-10
Gabbay 2012 (61)	T1D	18 months 2000 IU cholecalciferol daily or placebo	66±16 nmol/l	152±54 nmol/l		No change in % Tregs				No difference in IL-12, TNFα, CXCL10 or IL-10, but close-to-significant increase of CCL2 after 12 months (not after 18 months)
Terrier 2012 (141)	SLE	4 weeks 100.000 IU cholecalciferol weekly, then 6 months 100.000 IU monthly (no placebo group)	47±17 nmol/L	129±35 nmol/L		No change in total % or #. Increase in # naive at 6 months, but not %. No change in other activation stages. Increase in % and # of Tregs, aTregs and rTregs. Increase of % $CTLA4^+$ and $GITR^+$, but not LAP^+ Tregs. Decrease in % of Th1 and Th17 at 2 months, but only of Th1 at 6 months. No change in Th2.	No change in total % or #. Decrease in % effector memory at 2 and 6 months, but not #. No change in other activation stages. Decrease in $IFN\gamma^+$ at 2 months.	Decrease in % and # after 2 months, but after 6 months only in %. Increase in MZ % and # after 6 months. Decrease in % and # DN after 6 months. No change in naive or CS B cells	No change in % or # of NK cells	Anti-dsDNA decreased
Abou-Raya 2013 (66)	SLE	12 months placebo vs 2000 IU cholecalciferol daily	50±41nmol /L	95±41 nmol/L						Decrease in IL-1β, IL-6, IL-18 and TNFα Decrease in anti-dsDNA, anti-Sm and C4, but not anticardiolipin IgG or IgM
Piantoni 2015 (166), Andreoli 2015 (248)	SLE	12 months 25.000 IU cholecalciferol monthly (standard regime, SR) or 300.000 IU at baseline followed by 50.000 IU monthly (intensive regime, IR), compared with healthy control immune parameters	SR: 79 (20-211) nmol/L IR: 80 (47-188) nmol/L	SR: 68 nmol/l IR 96 nmol/l		Upon SR increase in % and [ ] of iTreg but not tTreg. In IR increased % iTreg and %tTreg, but not [ ]. In SR and IR increase in [ ] highly experienced Tmem, but only in % in SR. Increase in total CD4 % in SR and IR, but only in [ ] in IR. No change in % of $IL\text{-}17^+$, $IFN\gamma^+$ or $IL\text{-}4^+$ $CD4^+$ T cells after SR and IR.	Increase in % but not [ ] of $CD8^+$ in SR and IR. No change in % of $IL\text{-}17^+$, $IFN\gamma^+$ or $IL\text{-}4^+$ $CD8^+$ cells after both SR and IR, but in IR a decreased IFNγ/IL-4 ratio			No difference in anti-dsDNA between SR and IR

Figure 1| Vitamin D metabolism. The metabolic pathway of vitamin D. Red arrows indicate inhibition, green arrows indicate induction.

Figure 2| The anti-inflammatory effects of $1,25(OH)_2D_3$ on cells of the immune system. An overview of the anti-inflammatory effects of $1,25(OH)_2D_3$ on the cells of the immune system in autoimmunity. Red dots represent pro-inflammatory cytokines, while green dots represent anti-inflammatory cytokines. Red arrows indicate decreased differentiation, green arrows indicate increased differentiation. References: $CD8^+$ T cells (201, 202, 204); ILC (203, 211-213, 220); Unconventional T cells (144, 161, 208); B cells (75, 133-136, 138, 139, 143); DC (85-87, 91, 93-95); Macrophages (115, 125-128); $CD4^+$ T cells (141, 150, 155, 159, 163-167, 177-182, 184, 194).

Figure 01.TIF

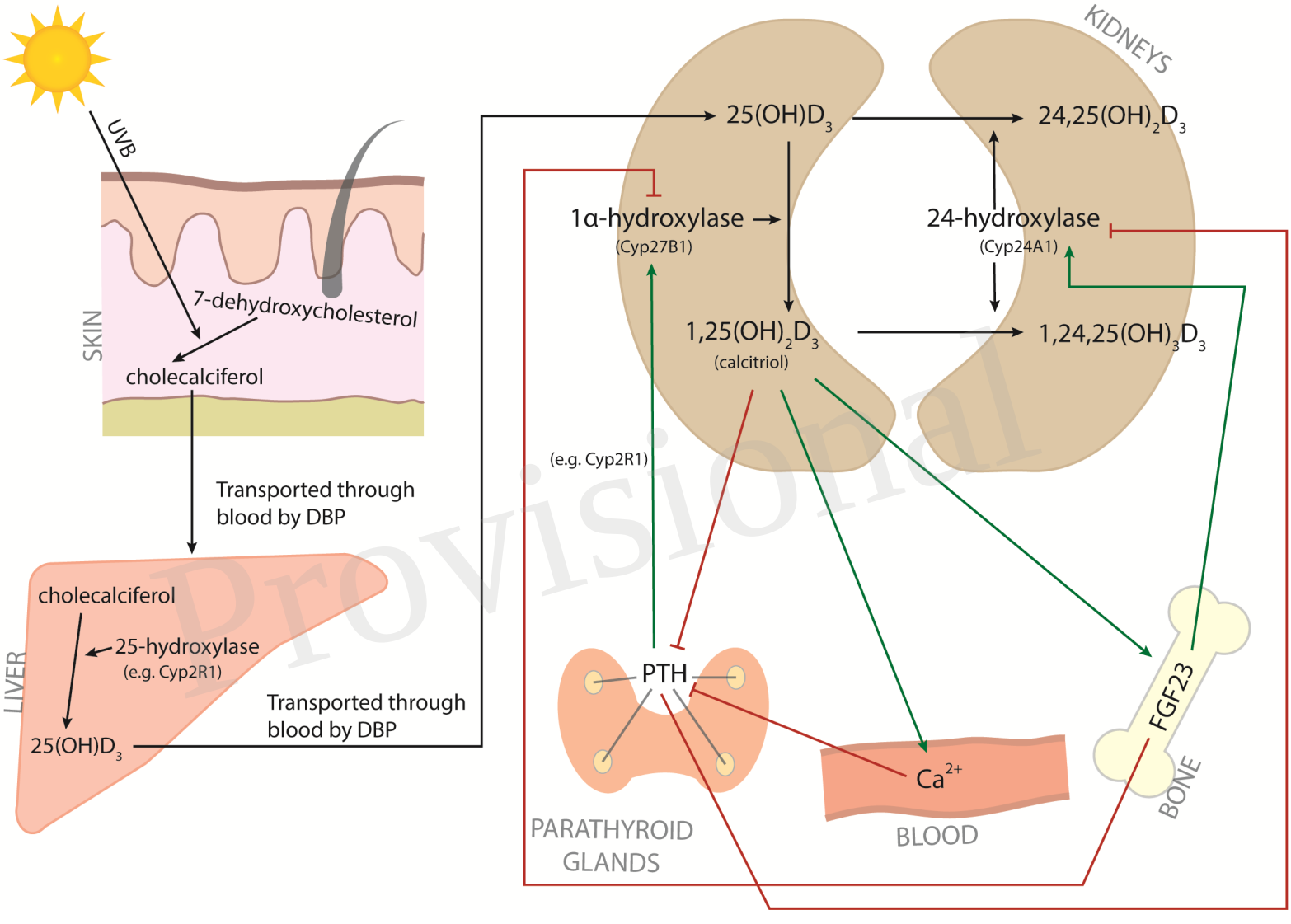

Figure 02.TIF