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Immunological effects of vitamin D and their relations to autoimmunity

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ABSTRACT

Vitamin D deficiency is an established risk factor for many autoimmune diseases and the anti-inflammatory properties of vitamin D underscore its potential therapeutic value for these diseases. However, results of vitamin D3 supplementation clinical trials have been varied. To understand the clinical heterogeneity, we reviewed the pre-clinical data on vitamin D activity in four common autoimmune diseases: multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD), in which patients are commonly maintained on oral vitamin D3 supplementation. In contrast, many pre-clinical studies utilize other methods of manipulation (i.e. genetic, injection). Given the many actions of vitamin D3 and data supporting a vitamin D-independent role of the Vitamin D receptor (VDR), a more detailed mechanistic understanding of vitamin D3 activity is needed to properly translate pre-clinical findings into the clinic. Therefore, we assessed studies based on route of vitamin D3 administration, and identified where discrepancies in results exist and where more research is needed to establish the benefit of vitamin D supplementation.

1. Introduction

Systemic autoimmune diseases result from interactions between an individual's genetic predispositions and environmental exposures. Vitamin D has long been considered an important contributing factor for human health, and the majority of studies exploring vitamin D metabolism and disease prevalence support a link between low serum vitamin D levels and autoimmunity [1,2]. For example, the African American population has historically been shown to have low vitamin D levels [3] and is 2–8 times more likely to develop Systemic Lupus Erythematosus (SLE) than the Caucasian populations in both the United States and United Kingdom [4]. Additionally, the prevalence of multiple sclerosis (MS) and inflammatory bowel disease (IBD) roughly follows a latitude gradient, with greater prevalence associated with increased distance from the equator, and consequently reduced sun exposure and lower vitamin D levels [1]. Also at the genetic level there is support for a correlation between low vitamin D and autoimmunity as mutations in genes involved in vitamin D transport, metabolism, or transcriptional activity of the vitamin D receptor (VDR) have been associated with increased incidence and/or severity in many such diseases [5–10]. Despite this overwhelming circumstantial evidence for a role for vitamin D in autoimmunity, several clinical trials surprisingly showed limited or no benefit of oral vitamin D supplementation, questioning the proposed cause-effect relationship between vitamin D levels and disease manifestations [11–15]. What is most noticeable in these studies is the discrepancy between the benefit of equatorial geographic location associated with increased sun exposure, and the relatively indifferent effect of oral supplementation observed in clinical trials. In this review, we briefly introduce the production and regulation of vitamin D and the VDR, before we discuss the impact of studies showing that manipulation of serum vitamin D3 levels, either genetically or applied, affect disease development and/or progression in animal models of four common autoimmune disorders. Delineating the immunologic properties of vitamin D in animal models may help us understand the discrepancies observed in clinical trials and aid in patient treatment.

2. Vitamin D3 metabolism

The vast majority of vitamin D is obtained through cutaneous synthesis in the form of vitamin D3, though a number of foods provide additional vitamin D2 (e.g. fungi) and D3 (e.g. fish, eggs) through intestinal absorption. Cutaneous production of vitamin D3 occurs in the plasma membrane of keratinocytes where 7-dehydrocholesterol undergoes photolysis into previtamin D3 upon exposure to ultraviolet B radiation from the sun (Fig. 1). Previtamin D3 is thermodynamically

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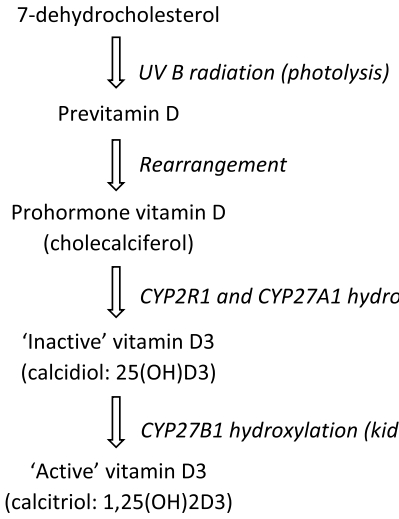

Fig. 1. Cutaneous production of vitamin D3. In keratinocytes, 7-dehydrocholesterol undergoes photolysis and rearrangement into cholecalciferol (vitamin D3). Cholecalciferol has minimal activity and must be hydroxylated first in the liver to 25(OH)D3, and secondly in the renal tubules to the hormonally active vitamin D3 derivative, 1,25(OH)2D3.

unstable and rapidly rearranges to the prohormone vitamin D3 cholecalciferol, which is then extruded from the cell membrane. Vitamin D3 is the primary form of vitamin D stored in tissues, predominantly the liver and adipocytes [16].

As a prohormone, vitamin D3 requires subsequent hydroxylations to assume its active hormonal state. Prohormone vitamin D3 is first hydroxylated in the liver to 25(OH)D3 (calcidiol) by CYP2R1 (and possibly also CYP27A1) [17,18]. 25(OH)D3, has a half-life of approximately 2 weeks and is the primary circulating form of vitamin D3 [19]. Eighty-five to 90% of circulating 25(OH)D3 is bound to the vitamin D binding protein, the remaining is either free or bound to albumin [3]. The biologically active vitamin D3 (1,25(OH)2D3; calcitriol) is formed by a second hydroxylation of 25(OH)D3 catalyzed by CYP27B1 (1α-hydroxylase). This process takes place predominantly in the proximal tubules of the kidney, although extra-renal CYB27B1 activity has also been shown [20].

The metabolism of active vitamin D3 (1,25(OH)2D3) is tightly regulated by feedback circuits. For example, low levels of circulating calcium trigger increases in parathyroid hormone (PTH), which in turn induces CYP27B1, raising 1,25(OH)2D3 levels and leading to increased calcium absorption in the intestines and reabsorption at the renal tubules [21]. Reciprocally, 1,25(OH)D3 directly inhibits transcription of PTH which prevents excessive vitamin D3 activity [22]. Degradation of 1,25(OH)2D3 is initiated by yet another hydroxylation step catalyzed by CYP24A1 [23], which is upregulated in response to 1,25(OH)2D3. Moreover, 1,25(OH)2D3 exerts negative feedback by downregulating CYP27B1 in certain cell types including the renal proximal tubule where CYP27B1 has the greatest expression [24]. Finally, fibroblast growth factor 23 (FGF23) is a phosphaturic hormone that inhibits CYP27B1 and induces CYP24A1 activity in renal tissue, thus representing another level of vitamin D3 regulation [25,26]. Given this tightly regulated control of serum active vitamin D3 levels, it is perhaps not surprising that dysregulation has been associated with disease, although the specific nature of any such cause-effect relationship remains unknown.

3. Vitamin D receptor (VDR)

1,25(OH)2D3 mediates transcriptional activity through its interaction with the VDR with an affinity of less than 1 nM [27]. The VDR is a member of a nuclear receptor superfamily characterized by two highly

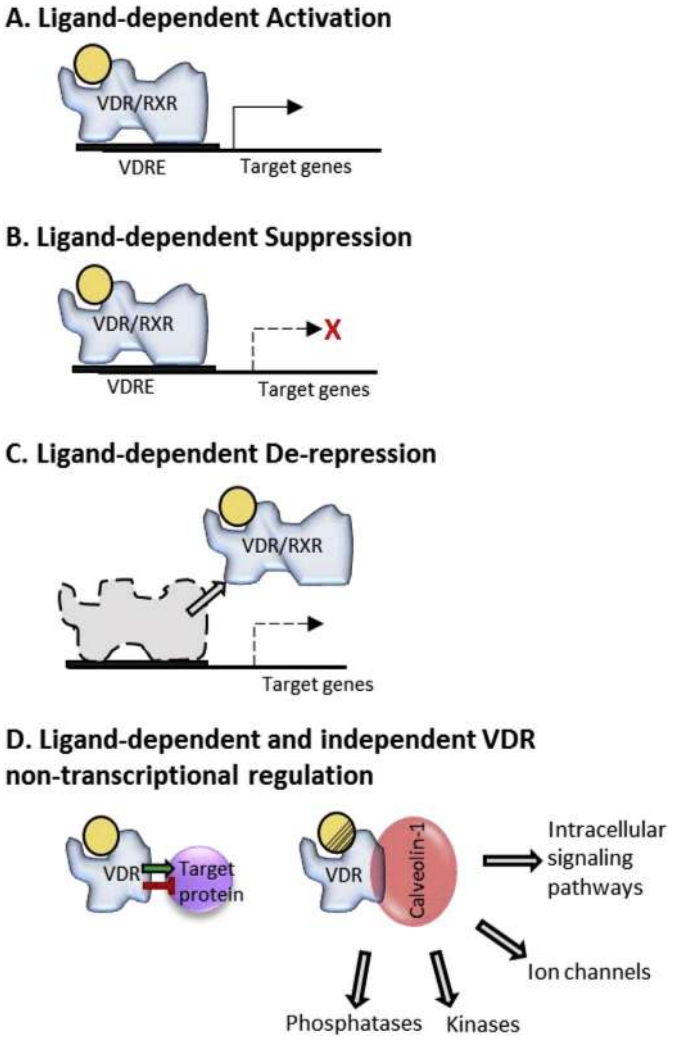

Fig. 2. Ligand-dependent and -independent actions of VDR. A) Ligand-dependent activation of target gene transcription. 1,25(OH)2D3 binds the vitamin D receptor (VDR) which complexes with Retinoid X receptor (RXR) and binds the vitamin D response element (VDRE) near target genes. The necessary transcriptional machinery is then recruited for initiation of gene transcription. B) Ligand-dependent suppression of target gene transcription. 1,25(OH)2D3 and the VDR/RXR complex inhibit gene transcription by recruitment of co-repressor molecules or blocking other transcription factor activity. C). Ligand-dependent de-repression. 1,25(OH)2D3 triggers release of bound, transcriptionally inactive VDR/RXR resulting in the availability for the transcriptional machinery and active gene transcription. D) Both ligand-dependent and -independent mechanisms of non-transcriptional protein regulation. On the left, ligand-bound VDR is capable of activating or inhibiting target proteins via direct binding. On the right, ligand-bound or ligand-free VDR has effects on intracellular signaling pathways, ion channels as well as kinase and phosphatase activity. For a review on non-genomic effects of the VDR, please see Hii et al. [31].

conserved domains: a central DNA binding domain containing two conserved zinc fingers, and a ligand binding domain in the C-terminus [28]. VDR complexes with the retinoic X receptor (RXR), and together the VDR/RXR complex binds DR3-type vitamin D response elements (VDRE) located upstream or downstream of VDR primary target gene promoters (Fig. 2) [29]. Binding of the VDR/RXR complex to VDRE subsequently recruits co-activators or co-repressors, which assist in transcriptional regulation by either 1) assembling the transcriptional machinery, 2) interfering with transcription via tethering of other transcription factors, or 3) driving epigenetic modifications via coactivator/corepressor-dependent associations with histone modification enzymes (Fig. 2) [30,31].

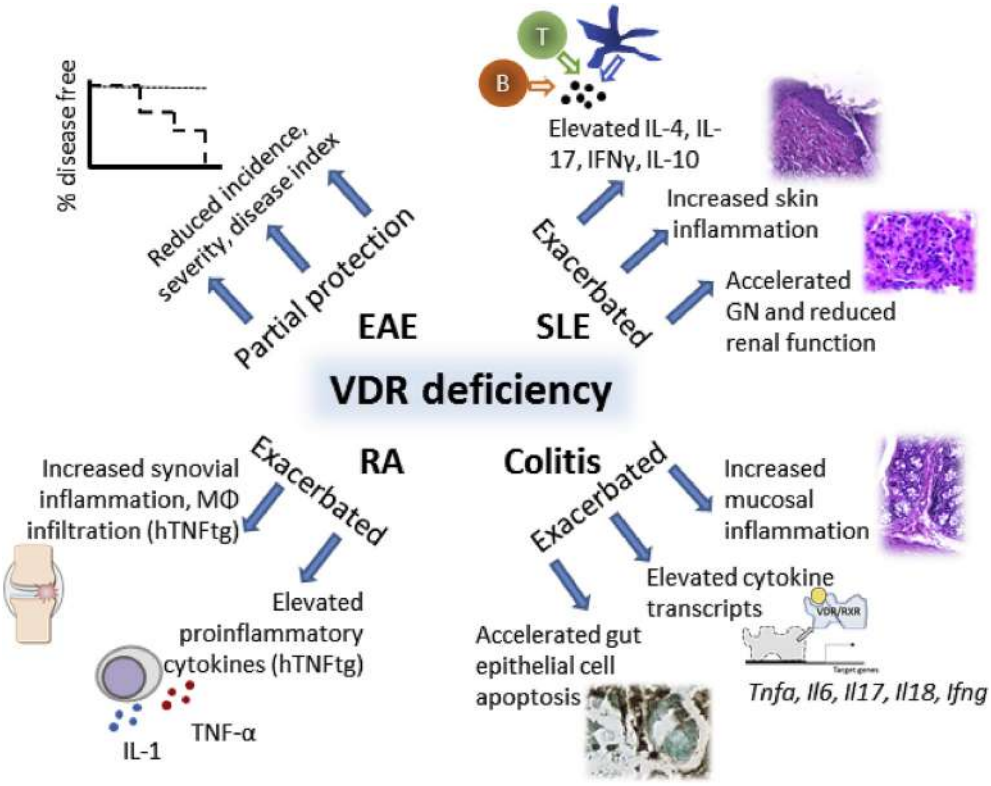

Fig. 3. The effect of genetic deficiency of VDR on autoimmune pathology in EAE, SLE, RA, and colitis disease models. VDR-deficient animal models of SLE, RA and colitis develop exacerbated autoimmune phenotypes including worsened histopathology and elevated markers of inflammation. In contrast, VDR-deficient EAE models are partially protected. VDR; vitamin D receptor. GN; glomerulonephritis. hTNFtg; human TNF transgenic arthritis model.

Although VDR-responsive genes are scattered throughout the genome, VDR-responsive genes are regulated in a tissue-specific manner in part due to tissue-specific differences in VDR expression/activity. This is exemplified by ChIP-seq studies, which identified 1600–2700 VDR-specific peaks across multiple tissue types [1,32,33]. Despite similarities in the total number of VDR-specific peaks across tissues there was only a 20% gene congruency, highlighting the presence of tissue-specific regulation of individual genes [30]. Interestingly, these data also identified "de-repression" patterns, in which a main VDR-specific peak was only observed in the absence of 1,25(OH)2D3 binding to the VDR/RXR complex. Based on these data, Carlberg et al. predicted that up to 10% of all 1,25(OH)2D3-responsive gene targets may operate under de-repression, further complicating the biological impact of dysregulated vitamin D3 levels [30].

In addition to transcriptional regulation in the nucleus, the VDR is also expressed at non-nuclear sites where it is believed to mediate non-genomic activities affecting ion channels and intracellular signaling cascades (Fig. 2; reviewed in Ref. [31]). For example, the VDR may localize to lipid raft calveolae-enriched plasma membranes and facilitate activation of c-Src and PLA2 signaling pathways [31,34]. Additionally, 1,25(OH)2D3 has been found to potentiate voltage gated chloride (CLC-type outwardly rectifying chloride channel) and calcium ion channels (L-type) in a VDR-dependent manner [35], while promoting PP1c and PP2A phosphatase activity via binding to VDR-phosphatase complexes and releasing the catalytic sites affecting proliferation and differentiation of colon cancer and leukemia cell lines [36,37]. Finally, multiple kinases (Ser-Thr/Tyr kinases, PI3K, PKA), phospholipases (PLC, PLA2), and other target proteins (e.g. IKKβ, STAT1, Runx1, c-jun, β-catenin) have been shown to be activated or inhibited by 1,25(OH)2D3 [31].

Expression of the nuclear VDR has been identified in monocytes, macrophages, dendritic cells and activated T and B lymphocytes consistent with evidence that vitamin D3 mediates differentiation and function of immune cells [38–41]. Although evidence is emerging in support of VDR-independent or non-classical VDR-dependent effects on immune cells, further studies are needed to address the clinical consequences of these pathways.

4. Manipulation of vitamin D3 levels in animal models of autoimmunity

Animal models of autoimmune diseases have provided significant insight into the relationship between vitamin D3 and disease initiation and progression. Over the past three decades, the effect of vitamin D3 has been studied in multiple animal models of autoimmunity via either genetic or physiologic manipulation. Genetic manipulation of VDR expression or vitamin D3 metabolic enzymes (CYP-family members) have been particularly important in driving our understanding of the critical role vitamin D3 plays in maintaining health, while physiological studies in which access to vitamin D3 has been augmented or restricted have provided valuable insight into the biological effects of smaller fluctuations. Here we will highlight the effect of vitamin D3 manipulation in animal models of four common autoimmune diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and inflammatory bowel disease (IBD), individually discussing results from studies using genetically engineered mouse models and supplementation/restriction.

4.1. Genetically engineered models of restricted vitamin D activity

4.1.1. Genetic manipulation of vitamin D and VDR in experimental autoimmune encephalomyelitis (EAE)

Given the well-accepted association between sun exposure and MS prevalence, studies have been undertaken to understand the role of vitamin D3 via VDR-dependent and –independent immune regulation in animal models of MS (EAE). Analyses in bone marrow chimeric mice lacking VDR expression in all hematopoietic cells or in T cells only, showed that expression of VDR in immune cell subsets, in particular T cells, is absolutely required for a protective effect by vitamin D supplementation (section 4.2.1 and 4.3.1) [42]. Contrary to the expectation then, VDR-deficient animals were in several studies shown to be at least partially protected from MBP- or MOG-induced EAE (Fig. 3)

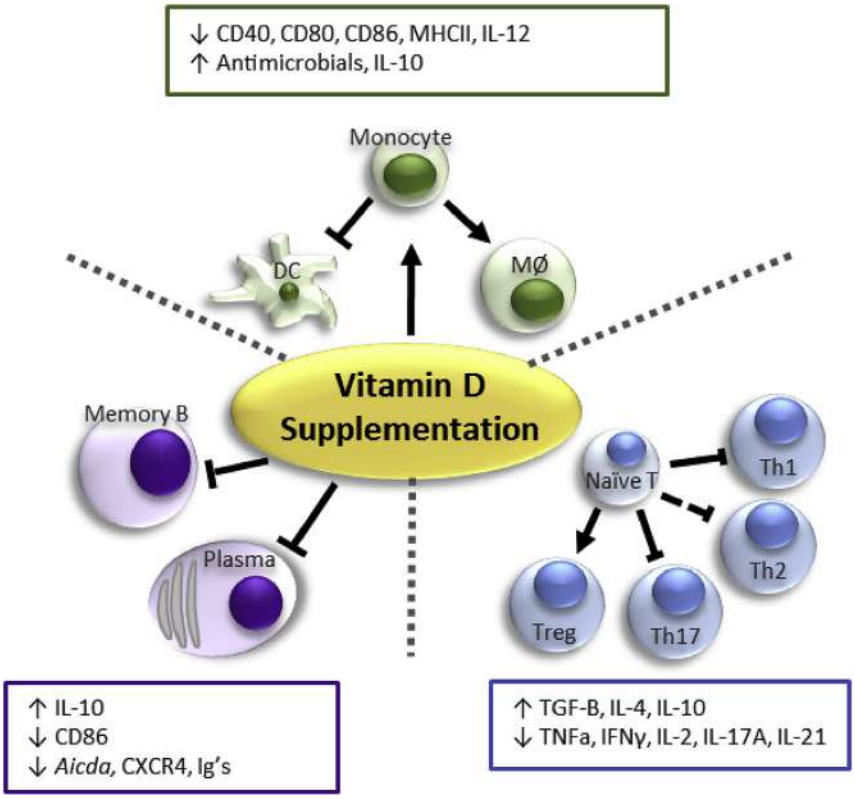

Fig. 4. Effects of vitamin D supplementation on monocyte, naïve T, and B cell differentiation and function. Vitamin D promotes differentiation from monocyte to macrophage and impairs activation of macrophages, dendritic cells and B cells. Naïve T cell differentiation in the presence of vitamin D favors development of T regulatory cells, and the production of TGF-β, IL-4, and IL-10, while suppressing the production of proinflammatory cytokines.

[42–44]. The reason for this discrepancy remains unknown. Hypercalcemia is an expected response to excessive vitamin D3/VDR activity, and studies have shown that high calcium in itself can prevent EAE in animal models [44,45]. This suggests that the therapeutic benefit observed in supplementation studies (see section 4.2.1 and 4.3.1) could be driven by vitamin D3-driven hypercalcemia, whereas loss of VDR may confer protection via other hypothesized mechanisms including altered hematopoiesis influencing the immune competency of VDR-deficient animals or upregulation of an unknown compensatory mechanism, however to date neither explanation has been further investigated. Finally, because the VDR can also exert inhibitory functions in the absence of vitamin D3 (e.g. the repressive function of VDR/RXR), loss of VDR can reveal binding sites and may promote the transcription of otherwise repressed genes [32]. It should also be noted that CYP2R1- or CYP27B1-deficiency had no effect on incidence and severity of EAE symptoms after immunization with MOG peptide [44,46], offering further support to this theory in which the protective effect is VDR-dependent, but ligand-independent.

4.1.2. Genetic manipulation of vitamin D and VDR in animal models of arthritis

Rheumatoid arthritis is similarly associated with vitamin D3 deficiency and VDR polymorphisms have been linked with increased risk of RA or RA severity in several studies [47–49]. The most commonly used rodent models of RA in vitamin D studies are induced either by collagen (CIA) or adjuvant (AA). Symptoms in these models largely mimic the human disease including joint swelling and proinflammatory serologic profiles [50]. Gu et al. showed that CYP27B1−/− CIA rats had more severe arthritis than WT rats as measured by gross phenotype, serologic, and imaging analyses [51]. Interestingly, i.p. treatment with 1,25(OH)2D3 improved all disease parameters as compared to vehicle-treated CYP27B1−/− littermates, proving that the effect was specifically due to active 1,25(OH)2D3 and not 25(OH)D3 [51]. Furthermore, 1,25(OH)2D3 treatment of CYP27B1−/− rats with CIA induced apoptosis of fibroblast-like synoviocytes in a VDR-dependent manner, reducing the local destruction of articular cartilage caused by TNFα-induced expansion of fibroblast-like synoviocytes [51]. Although, no study has specifically investigated the role of the VDR in CIA mouse models, this single study highlights the importance of 1,25(OH)2D3 and VDR signaling in CIA. Additional studies are needed to confirm these findings and to elaborate on VDR-dependent apoptosis as a possible mechanism to explain vitamin D3 benefits in CIA and the potential role for VDR-independent effects.

Aside from CIA and AA, genetic models of RA have been generated including the hTNFα-transgenic mouse model and the K/BxN model, both of which spontaneously develop arthritis [50]. In correlation with a protective role for vitamin D3 in RA, VDR-deficiency in hTNFα transgenic models resulted in increased disease activity and elevated synovial inflammation marked by an influx of macrophages and an increase in proinflammatory cytokine transcripts (Fig. 3) [52]. Studies evaluating the effect of CYP-deficiencies and/or vitamin D3 supplementation/restriction in this model have not been done, neither has the role of vitamin D3 in the K/BxN model been investigated.

4.1.3. Genetic manipulation of vitamin D and VDR in animal models of SLE

Vitamin D3 deficiency has been linked to SLE in multiple clinical studies [53,54]; however, to our knowledge only a single study has briefly investigated a possible role of genetic VDR-deficiency in a mouse model of lupus and no study has yet evaluated the effect of genetically modified CYP-family members. Using VDR-deficient MRL mice, Ding et al. recently reported accelerated immune cell dysregulation, increased IL-4, IL-17, and IFN-γ levels, increased skin inflammation and glomerulonephritis, and reduced renal function, supporting a protective role for VDR in the development of lupus-like features (Fig. 3) [55]. Surprisingly, IL-10 levels also increased over time in VDR-deficient MRL mice [55] suggesting that Tregs, Bregs and/or tolerogenic DCs could be involved. This is in contradiction to many other studies where the addition of vitamin D3, not VDR deficiency, has been shown to promote Treg, and tolerogenic DC differentiation or function and IL-10 production (Fig. 4) [56–59], the latter specifically via direct

upregulation of the Treg-associated transcription factor Foxp3 by VDR [59]. Further studies in other mouse models of SLE are needed to determine if such profile is general for this disease or specifically present in MRL mice.

4.1.4. Genetic manipulation of vitamin D and VDR in animal models of colitis

There is considerable evidence for a role of vitamin D3 through VDR manipulation studies in animal models of colitis (Fig. 3). Global VDR deficiency increases susceptibility to DSS-induced colitis [60], and VDR/IL-10 double knockout mice develop an accelerated colitis with 100% mortality rate after only 8 weeks as compared with single IL-10- or VDR-knockout animals that remain relatively healthy at that time point [61]. Furthermore, transgenic overexpression of the human *VDR* gene in intestinal epithelial cells, rescues the colitis phenotype of not just IL-10-deficient mice, but also of TNBS-induced colitis in global VDR−/− mice, resulting in reduced mucosal inflammation, decreased immune cell infiltration, decreased apoptosis, and decreased levels of proinflammatory cytokine mRNAs (*Tnfa*, *Il6*, *Il17*, *Il18* and *Ifng)* [62,63]. These findings mirror data from other *in vivo* as well as *in vitro* studies that support vitamin D3's both direct and indirect transcriptional regulation of signature cytokines (e.g. IL-2, IL-4, IL-17A, IFNγ) [59,64–66]. Interestingly, intestinal epithelial cell-specific VDR deficiency also resulted in colitis with aggravated epithelial cell apoptosis, suggesting that VDR expression in these cells is particularly critical for maintaining gut health [63]. Importantly, transfer of $CD45RB^{hi}$ cells from VDR−/− to Rag−/− mice also causes severe colitis attributable to the lack of VDR expression in lymphocytes specifically [61]. Collectively these results support a two-fold role for vitamin D3 in colitis pathogenesis: 1) vitamin D3/VDR signaling in the intestinal epithelium is important in maintaining intestinal health possibly by mediating epithelial apoptosis, and 2) VDR-deficient splenocytes from colitic mice are pathogenic. Further studies using CYP-specific knockout/knockdown models are needed to determine if the effect is vitamin D3-dependent or –independent.

4.2. Intraperitoneal vitamin D supplementation

4.2.1. Vitamin D3 administered by i.p. injection in EAE

Two decades of research have demonstrated that vitamin D3 supplementation by i.p. injection improves multiple clinical endpoints in EAE mouse models of multiple sclerosis (Table 1) [67–70]. In summary, injection of vitamin D3 results in reduced incidence [67,70], improvement in hind limb paralysis/clinical scoring, reduced relapse rate [67], and overall increased survival [67]. In addition, 1,25(OH)2D3 supplementation has been associated with reduced perivascular inflammation, white matter and meningeal inflammation [67], and demyelination/axonal loss [70]. Thus, vitamin D3 treatment via i.p injection is effective in treating and preventing both symptomatic and histological aspects of EAE.

4.2.2. Vitamin D3 administered by i.p. injection in animal models of arthritis

In CIA models of RA, vitamin D3 administered by i.p. injection was found to be anti-inflammatory and to suppress arthritis onset and/or severity regardless of differences in induction protocol, source and amount of collagen II, timing of induction, and the source and dose of vitamin D3 (1,25(OD)2D3 or 20-epi-1a-(OD)2D3) [51,71]. Importantly, reduction in incidence and joint swelling was noted in both prophylactic protocols and upon treatment of established disease [51]. Additionally, at lower, non-calcemic doses of vitamin D3, there was a significant reduction in IgG2a antibody levels; the predominant complement fixing immunoglobulin important in the pathogenesis of CIA [72]. An effect on immunoglobulin levels is also supported by *in vitro* studies in which naïve B cells exposed to vitamin D3 were less likely to differentiate into antibody secreting plasma cells (Fig. 4) [73–75] and studies in healthy individuals showing that low serum 25(OH)D3 was positively associated with IgG2 [76], and inversely correlated with IgG1 and IgA [76,77]. The fact that *Aicda* expression is inhibited in B cells after 48 h of incubation with 1,25(OH)2D3 further suggests that vitamin D3 interferes directly with the Ig class switching process (Fig. 4) [75].

In contrast to the studies on CIA, two studies on AA reported conflicting results. Boissier et al. showed that i.p. injection of 1,25(OH)2D3 reduced the arthritis score without an effect on incidence [78]. In contrast, Oelzner et al. concluded that there was no effect of 1,25(OH)2D3 injection on knee joint swelling or histopathology [79]. This discrepancy may be due to the fact that the two studies used animals of different sex and strain, as well as different injection protocols (injections daily from day −3 to +15, then every other day to +28, compared to daily from day 0 to +13). Future studies directly comparing the effect of vitamin D3 on both male and female rats of similar genetic backgrounds using comparable immunization/induction protocols are needed for formal conclusions to be made.

4.2.3. Vitamin D3 administered by i.p. injection in animal models of SLE

Similar to the lack of studies in VDR- and CYP-deficient mice susceptible to lupus, only a few studies have been performed investigating the efficacy of i.p. injected vitamin D3. First, male MRL/MP-lpr/lpr mice injected with a synthetic vitamin D3 analog, 1,24(OH)2D3, displayed improved survival and reduced proteinuria [80]. Secondly, female MRL/lpr treated i.p. every other day with active 1,25(OH)2D3 on a low-to-normal calcium diet were protected from the characteristic dermatologic lupus phenotype, and furthermore showed reduced serum anti-nuclear autoantibody (ANA) levels and reduced urine protein-to-creatinine ratio [81].

A single study assessed the effect of i.p. administration of the cholecalciferol in the NZB/W mouse model of lupus, but found no evidence of a protective role [82]. This might be attributed to the use of cholecalciferol rather than active 1,25(OH)2D3, as the latter has been shown to be important in the treatment of CIA [51], although further studies are needed to address this possibility. Also, although the results from the MRL/lpr and NZB/W models appear to be at odds with each other, it is important to remember that while the MRL/lpr model is largely driven by T cells, the NZB/W model of lupus relies heavily on B cell dysregulation and the production of autoantibodies. And while there is evidence to support a possible role for 1,25(OH)2D3 in impairing B cell proliferation [74,83,84] and class switching/germinal center reactions [75,85], and an association between 25(OH)D3 level and circulating immunoglobulins [76,77], the observed effects are not as strong as the

Table 1
Studies of animal models of autoimmunity by vitamin D intervention.

Disease	Genetic manipulation		Physiologic availability			References
	VDR−/−	CYP -deficiency	i.p. treatment	Oral supplementation	Oral restriction	
EAE	↓	↑	↓	↓	↓*	GM: 42–44, 46; PA: 67–70, 94–95, 101-102
RA	↑	↑	↓↔	↓	↑	GM: 51–52; PA: 51, 71–72, 78–79, 103-104
SLE	↑	nd	↓↔	↓	nd	GM: 55; PA: 80–82, 109-110
Colitis	↑	nd	↓↔	↓↔	↑	GM: 60–63; PA: 100, 111-114

nd: not done; * generational vitamin D depletion; GM: genetic manipulation; PA: physiologic availability. ↑ Exacerbated; ↓ improved; ↔ no effect.

paramount data supporting effects on T cells (Fig. 4). Taking advantage of newer well-defined mouse models of SLE in which the contribution from different immune compartments is controlled, may be important to determine if vitamin D3 supplementation has an impact on lupus-like disease development.

4.2.4. Vitamin D administered by i.p. injection in colitis

Only a single study has assessed the effect of i.p. administration of vitamin D treatment in models of colitis using an analog of vitamin D2. Intraperitoneal injection of paricalcitol (19-nor-1,25(OH)2D2 at 0.5 μg/kg body weight) administered 30 min prior to induction of colitis reduced colon damage and apoptosis, as well as preserved tight junction function and intestinal integrity in TNBS-induced colitis, but not oxazalam-induced colitis [86]. TNBS-induced colitis and oxazalam-induced colitis are models for Crohn's disease and Ulcerative Colitis, respectively. Differences in colitis pathophysiology likely determines responsiveness to vitamin D2 treatment and suggests that vitamin D might be of specific benefit in Crohn's disease (Th1/Th17 driven) rather than Ulcerative Colitis (Th2 driven). This pattern is consistent with observations that vitamin D3 inhibits T cell differentiation into Th1 and Th17 subsets, while the effect on Th2 differentiation and *Gata3* gene expression is less clear (Fig. 4) [87–90].

4.3. Oral vitamin D3 supplementation/restriction

Recently, studies have identified associations between the gut microbiome and autoimmunity [91,92]. Furthermore, it has been shown that bacterial colonization influences vitamin D3 metabolism. Germ-free mice were found to express high levels of FGF23, which is known to suppress the hydroxylation of 25(OH)D3 by CYP27B1 [93]. As a result, germ-free animals display low serum calcium levels and low active vitamin D3, both of which are significantly increased upon conventionalization [93]. These recent pieces of evidence suggest an intimate relationship between the gut and vitamin D3, therefore the method of vitamin D3 acquisition (oral vs. injection vs. skin) may be of importance. In this section we discuss the results of oral supplementation and restriction of vitamin D3 in the same four autoimmune diseases presented above.

4.3.1. Oral supplementation/restriction of vitamin D3 in EAE

In addition to the abundance of studies evaluating genetically manipulated models of EAE as described (section 4.1.1), there are many reports of oral supplementation of vitamin D3 in mouse models of MS. When compared to oral restriction, a number of studies supported a protective role for supplementation with oral cholecalciferol [94] and 1,25(OH)2D3 supplementation (Table 1) [42,43,94–96]. Overall, these studies showed significant reduction in clinical disease activity index, with one study reporting complete prevention of disease onset in response to supplementation [94]. These are robust data as the dietary supplementation concentration ranged from 20 ng/day to 1 μg/day and the data were generated over the span of two decades. It should be noted though, that these studies compared vitamin D3 restriction against a higher amount of vitamin D3 than is considered “normal”, rather than versus normal vitamin D3. Although vitamin D restriction is anticipated to exacerbate autoimmunity, because the available data compares restriction to supplementation, the effect of vitamin D restriction compared to normal intake remains unclear.

Seemingly contradictory, it has also been reported that vitamin D3 deficiency is associated with a delay in EAE onset and severity when EAE is induced in offspring from vitamin D3 deficient breeders [44,97,98]. In one study, supplementation with high levels of calcium [44] was also protective against disease severity, although further studies are needed to evaluate if calcium dysregulation is the primary mechanism of protection or if generational depletion of vitamin D3 might lead to epigenetic changes in vitamin D3 metabolism or VDR expression. Furthermore, long-term vitamin D3 depletion may affect immune system function, impairing the individual's ability to mount an autoimmune attack. Clinical studies in severe vitamin D3 deficiency in adolescents supports this idea [99].

Mechanistically, it was gathered from these supplementation studies, that IL-4 and IL-10 are key players in vitamin D3-mediated EAE improvement [95,100,101]. Specifically, the lack of response to oral vitamin D3 supplementation in both IL-4−/− and IL-10−/− mice, suggested a specific role for these cytokines in driving vitamin D3-dependent protection [100,101]. Also, STAT6, a protein required for IL-4-induced intracellular signaling, was required for symptomatic improvement in animals treated with a high-vitamin D3 diet [102], further supporting a role for IL-4 in vitamin D3-mediated disease protection. Regulation of IL-4 and IL-10 has also been proven *in vitro*, where vitamin D3 induces IL-10 production by promoting differentiation of naïve T cells into Tregs [59] and increases IL-4 secretion by Th2 differentiated cells (Fig. 4) [89]. Thus, in animal models of EAE, the route of vitamin D3 supplementation (i.p. versus oral) appears to be of minimal importance, while the effect is dependent on functional VDR-expression by T cells in particular.

4.3.2. Oral supplementation/restriction of vitamin D3 in animal models of arthritis

Two separate studies in animal models of RA showed a positive effect of vitamin D3 oral supplementation [103,104]. In the CIA model, mice fed a diet with 20–50 ng/day of active 1,25(OH)2D3 showed reduced arthritis scores and improved histopathology as compared to mice fed the same diet devoid of active 1,25(OH)2D3 [103], suggesting that supplementation with active 1,25(OH)2D3 offers protective benefit over standard maintenance. Surprisingly, we were unable to identify studies in which vitamin D3 restriction were investigated the CIA model.

In an adoptive transfer model of induced arthritis, rats receiving arthritogenic T cells and fed either vitamin D3-restricted (no vitamin D3), vitamin D3-supplemented (1 μg/day), or standard chow, showed that vitamin D3 supplemented recipient rats developed lower joint arthritis scores compared to recipients fed standard or vitamin D3 restricted chow [104]. Interestingly, the peak of clinical inflammation (9 days post-transfer) corresponded with an overall increased immune cell (activated monocytes, $CD4^+$ T cells, polymorphonuclear cells) infiltration into synovium-rich tissues and delayed inflammation resolution in the vitamin D3 restricted recipient rats. There were no cytokine analyses to support these findings, so whether vitamin D3 supplementation acted via modulation of T cell proliferation or T cell effector mechanisms (i.e. T cell activation, differentiation and cytokine production) remains unknown. It should be noted though, that in this adoptive transfer model vitamin D deficiency was induced in the recipient, rather than the donor, suggesting that vitamin D3 plays an important role in shaping the recipient's response to inflammation and less so in driving the pathogenicity of the effector T cells [104]. In support hereof, vitamin D3 has been shown to reduce proinflammatory cytokine production by myeloid cells [105], and to promote the development of tolerogenic DCs [57,106] and Tregs (Fig. 4) [59,107], both of which may dampen the pathogenicity of the transferred pathogenic T cells via secretion of IL-10. Alternatively, treatment with 1,25(OH)2D3 may have acted directly on the transferred effector T cells to prevent their expansion via inhibition of IL-2-dependent proliferation, as described *in vitro* (Fig. 4) [38,39,66,108].

4.3.3. Oral supplementation/restriction of vitamin D3 in animal models of SLE

Few studies have evaluated the effect of oral vitamin D3 supplementation in models of SLE and none have assessed dietary restriction in mouse models of SLE. Using the MRL/MPJ autoimmune model, DeLuca et al. showed that dietary supplementation with 1,25(OH)2D3 prevented proteinuria and reduced disease severity, when the mice also received a high calcium diet (0.87% Ca), but not when receiving a low

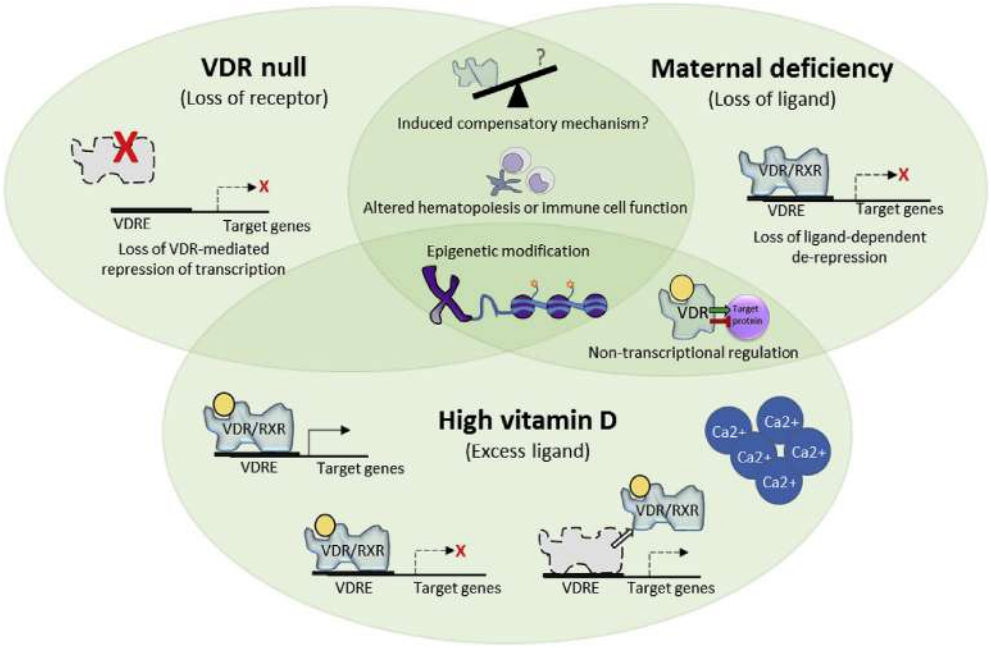

Fig. 5. Models of protection in the EAE mouse. Vitamin D supplementation (oral or i.p. injection), loss of VDR, and maternal vitamin D deficiency all provide at least partial protection against disease in the EAE mouse despite assumed contradictions between increased vitamin D/VDR signaling in vitamin D supplementation and decreased vitamin D/VDR signaling in the VDR null and maternal deficiency models. VDR null and maternal deficiency models may rely in part on a shared mechanism that involves altered immune cell development (e.g. compensatory mechanisms develop and changes in hematopoiesis and immune cell function occur), as well as on unique mechanisms dependent on the respective VDR status. In comparison, benefits in vitamin D supplementation are known to be dependent on the presence of both ligand and receptor. Non-transcriptional regulation is likely affected by manipulation of ligand availability in supplementation and maternal deficiency models, while epigenetic modification mechanisms dependent on VDR ± vitamin D may be shared between maternal deficiency and VDR null models. VDR: Vitamin D receptor; RXR: Retinoic X receptor; VDRE: Vitamin D Response Element.

calcium diet (0.02% Ca) [109]. This highlights the importance of calcium in vitamin D3-mediated therapy for autoimmune disease as was discussed in the EAE model (section 4.1.1). Similarly, oral administration of a vitamin D3 analog (22-oxa 1,25(OH)2D3) resulted in increased survival, reduced proteinuria, and diminished joint inflammation in the MRL/1 model of SLE [110], while in the MRL-lpr/lpr mouse, dietary 1,25(OH)2D3 supplementation over 24 weeks reduced IL-4, IL-10, IL-17 and IFNγ levels, as well as improved renal histopathology and complement deposition [55]. Further studies investigating the effects of vitamin D3 oral supplementation or restriction in additional mouse models of lupus-like disease are needed to establish the underlying protective mechanisms of vitamin D3 and/or non-symptomatic hypercalcemia in mouse lupus and to provide solid pre-clinical evidence to support prescription of vitamin D3 to SLE patients.

4.3.4. Oral supplementation/restriction of vitamin D3 in animal models of colitis

Although few studies have compared dietary vitamin D3 restriction against standard dietary intake, available data suggest that vitamin D3 deficiency exacerbates colitis in animal models. For example, a vitamin D3-deficient diet led to increased susceptibility to DSS-induced colitis in C57Bl6 mice [111] and increased mortality of IL-10 knockout mice [100] compared to mice receiving a standard diet or 5 μg cholecalciferol, respectively. In contrast to the paucity of studies evaluating vitamin D3 restriction, the protective effect of oral vitamin D3 supplementation is extensively described in mouse models of colitis. In a well-controlled study, male mice underwent 7 days of DSS treatment before being treated for 14 days with either 1,25(OH)2D3 or saline by oral gavage [112]. Mice treated with 1,25(OH)2D3 had longer colons, improved weight recovery, and showed overall improved disease activity and histological scores compared to control saline-treated mice [112]. While this study demonstrated the benefit of 1,25(OH)2D3 treatment after DSS-triggered colitis, data also suggest 1,25(OH)2D3 is beneficial as a preventative treatment when given 1 week prior to and throughout DSS treatment [113]. In contrast to observations in EAE and SLE models, mice with DSS-triggered colitis treated with 1,25(OH)2D3 did not consistently experience symptomatic improvement associated with higher serum calcium levels [112,113]. In the IL-10−/− model, IL-10−/− mice born to vitamin D3 deficient mothers and supplemented with 1,25(OH)2D3 (5 ng/day) for 4 weeks also demonstrated significant improvement in histology scores compared to those maintained on a vitamin D deficient diet [100]. Benefit was also observed if 1,25(OH)2D3 (0.2 μg/day) supplementation was initiated at the time of colitis onset (approximately 7 weeks of age) demonstrating beneficial treatment effects after clinical presentation [100]. Similarly, a diet supplemented with 25(OH)D3 was enough to significantly improve body weight and colonic histology scores in a chronic model of colitis driven by adoptive transfer of $CD4^+$ effector T cells from IL-10−/− mice [114]. These results suggest that the active form of vitamin D has both preventive and therapeutic benefit in animal models of colitis. Interestingly, the beneficial effect of vitamin D3 was apparent both when IL-10−/− T cells were driving the disease and in the IL-10−/− model of colitis itself, suggesting that vitamin D3 acts in an IL-10-independent manner and hence differently than in the EAE model in which IL-10 was shown to be critical for the vitamin D3-driven benefits [101].

5. Conclusion

The general impact of vitamin D3 on animal models of autoimmunity appears to be beneficial regardless of whether treatment is provided via oral or i.p. administration routes (Table 1). However, the available literature for each disease entity reviewed is limited and some contradiction exists, as discussed above (sections 4.2.2, 4.3.1, and 4.3.4). Differences reflecting unique pathologic mechanisms between diseases, animal models, sex, or methods of disease induction are common, thus highlighting the need for studies using comparable protocols.

Additionally, the versatile activity of the VDR (e.g. genomic, non-genomic, ligand-dependent, ligand-independent, tissue-specificity) suggests that the effects of high or low vitamin D3 may be more complicated to interpret. Using the EAE model as an example, these studies revealed interesting discrepancies between different combinations of vitamin D exposure and functional VDR status (Fig. 5). Notably, both

maternal vitamin D deficiency and loss of VDR confer partial protection against EAE, compared to vitamin D supplementation which has the capacity for full protection, but requires the presence of VDR. There are likely multiple mechanisms involved, and different mechanisms may be activated in ligand-dependent or -independent ways, as well as VDR-dependent and -independent ways. Elucidating further the individual mechanisms and the differences between them are needed before we fully understand the composite influence of vitamin D on autoimmunity. Furthermore, there appears to be differences between different autoimmune models and thus such studies should be done for each disease entity, as we suspect the mechanisms may differ between disease entities.

We observed considerable variability in the treatment groups being compared – low vs. normal/control, normal/control vs. high, or low vs. high vitamin D3 interventions – in the studies referenced above. It is worth noting that the interpretation of results depends on these treatment groups, and that the interpretation might differ between analyses of low vs. normal and low vs. high, for example. Given that vitamin D deficiency is viewed as a risk factor for autoimmunity, understanding the role for low vitamin D3 in autoimmune disease as compared to normal levels of vitamin D3 may be of specific interest for the medical community.

Finally, most supplementation studies use 1,25(OH)2D3 in their respective autoimmune models and the available results from these studies suggest that the active 1,25(OH)2D3 is important for protective function. However, 25(OH)D3 is commonly prescribed clinically for vitamin D3 deficiency due to the risk of hypercalcemia with 1,25(OH)2D3 treatment. At this time there is insufficient evidence to evaluate whether positive treatment effects of 1,25(OH)2D3 observed from animal studies are maintained using either cholecalciferol or 25(OH)D3. Moreover, data also support therapeutic benefits with high serum calcium levels in several models. Thus, additional studies are critical to understand the interplay between vitamin D status and autoimmunity, and whether supplementation with low doses of 1,25(OH)2D3 without symptomatic hypercalcemia, rather than the currently prescribed 25(OH)D3, is required for clinical efficacy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.03.002.

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