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Therapeutic and Prophylactic Potential of Vitamin D for Multiple Sclerosis

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Additional information is available at the end of the chapter

Abstract

A plethora of investigations demonstrated that vitamin D (VitD) has a broad immuno-modulatory potential. It induces tolerogenic dendritic cells *in vitro* leading to the development of regulatory T cells that have a key role in immunomodulation of autoimmune diseases including multiple sclerosis (MS). Studies showed that many MS patients present lower serum levels of VitD than healthy subjects. In addition, VitD supplementation has been associated with a reduced relative risk of developing MS. Considering the alterations in VitD levels in patients and also the immunomodulatory properties of VitD, it would be interesting to evaluate VitD potential as a tolerogenic adjuvant in experimental models of MS. In this context, our research team has been investigating strategies employing VitD to establish an *in vivo* tolerance state toward central nervous system antigens in experimental autoimmune encephalomyelitis (EAE). We observed that the association between a myelin peptide and VitD determined both therapeutic and prophylactic effects on EAE development.

Keywords: vitamin D, multiple sclerosis, experimental autoimmune encephalomyeli-tis, immunomodulation, myelin peptides

1. Introduction

The immune system is well known by its ability to defend the host against infections. In this sense, it is academically subdivided into innate and adaptive immune responses. Innate immunity is the first defense line and includes the microbicidal activity of macrophages and

polymorphonuclear cells. Host defense against microorganisms is dependent upon recognition of pathogen-associated molecular patterns, mainly by toll-like receptors (TLRs) present in these cells. Otherwise, adaptive immunity requires specific antigen recognition by B and T lymphocytes. Differently from B cells that can directly recognize the antigens, T cells require previous antigen processing and interaction of epitopes with major histocompatibility complex proteins that are then expressed at the surface of antigen-presenting cells (APCs) as, for example, dendritic cells (DCs). Due to their strong potential for proliferation and activation, B and T cell activity needs to be regulated. A special T-cell subpopulation called regulatory T (Treg) cell plays a major role in controlling inflammatory immune responses. To maintain its homeostasis, the immune system has to manage a balance between inflammatory and anti-inflammatory responses. The imbalance of these immune responses leads to the development of many diseases such as autoimmune pathologies. In this context, other T-cell subpopulations such as T helper type 1 (Th1) and type 17 (Th17) cells, which are inflammatory, and type 2 cells (Th2), which are predominantly anti-inflammatory, are also involved. Besides its ability to eliminate pathogens and restore the host homeostasis, the immune system has also a mechanism to hamper the development of an immune response against the body's own tissues. This mechanism, called self-tolerance, can be disrupted by the combination of a variety of genetic, environmental, and immunological factors that lead to autoimmunity. The relevance of vitamin D (VitD) in multiple sclerosis (MS), which is an autoimmune disease involving the central nervous system (CNS), is discussed in this chapter.

2. VitD metabolism

The history of VitD is strongly linked to rickets and its treatment with cod liver oil. In 1922, McCollum [1] coined the term vitamin D to refer to the antirachitic factor found in cod liver oil [2]. For this reason and for a long time, the most widely accepted physiological role of VitD was related to calcium and phosphorus metabolism and bone mineralization [3]. However, since the 1980s, many researches implicated VitD on the cardiovascular, endocrine, and central nervous system (CNS), as well as on the immune system physiology. The active form of VitD (1α,25-dihydroxyvitamin D_3) determines pleiotropic effects in human body through binding to vitamin D receptor (VDR), which is a member of the steroid hormone receptor superfamily found in a variety of human cells. The biological effects of VitD can be elicited by non-genomic and genomic mechanisms depending on the cell location of VDR. The non-genomic (rapid) mechanisms consist in VitD direct effect on the cells through membrane VDR binding. These effects include, for example, the activation of protein kinase C in different organs [4]. The genomic mechanism is determined by intracellular VDR that heterodimerizes with retinoic X receptor after binding to active VitD. This heterodimer is then translocated to the nucleus leading to activation or inhibition of a vast diversity of genes [5].

Some of the most important aspects of VitD epidemiology have been established by the scientist Michael Holick and his collaborators. As many people do not have an adequate

sunlight exposure due to skin cancer risk, sedentary lifestyle, darker skin, or during the winter in countries far from the equator, there is an increasing number of persons with VitD deficiency around the world [6]. In the past few years, VitD deficiency has been associated with the etiology of many chronic diseases, like Crohn's disease, infections of the upper respiratory tract, cancer, myocardial infarction, Alzheimer's disease, autoimmune diseases, and others [7]. According to current knowledge, VitD serum levels should be between 30 and 100 ng/mL in healthy humans. VitD insufficiency is related to levels between 21 and 29 ng/mL, whereas a pronounced VitD deficiency is considered in individuals whose VitD levels are below 20 ng/mL. On the other hand, serum levels over 150 ng/mL can determine intoxication VitD intoxication [8]. Excessive oral intake of VitD may cause a hypervitaminosis condition with toxic effects such as hypercalcemia and hypercalciuria. Theories concerning the mechanisms of VitD toxicity involve elevated plasma concentration of VitD itself or its metabolites that culminates in overexpression of a variety of genes [9]. Although solubility of vitamins (fat or water) has no direct effect on toxicity, the ability of fat-soluble vitamins such as VitD to accumulate in the adipose tissue determines their higher toxic potential than water-soluble vitamins. For example, subcutaneous fat necrosis releases tissue-accumulated VitD that leads to hypervitaminosis and its toxic effects [10].

The highest amounts of VitD are synthesized by the skin exposed to sunlight. Ultraviolet radiation converts 7-dehydrocholesterol in pre-vitamin D3. Then pre-vitamin D3 suffers a spontaneous thermal isomerization into vitamin D3, named cholecalciferol [11]. Due to this essential role of sunlight, this vitamin has been called "sunshine vitamin" [12]. Smaller amounts of VitD can be obtained from intake of certain foods such as mushrooms, fish, milk, and eggs [13]. To become a metabolically active hormone, cholecalciferol needs to be hydroxylated twice. The first hydroxylation takes place in the liver and converts cholecalciferol into 25-dyhidroxyvitamin D (calcidiol) via the enzyme 25-hydroxilase [14]. Plasma calcidiol levels are usually used as a parameter of VitD status because it increases in proportion to VitD intake [15]. After that, calcidiol binds to a carrier molecule, known as the vitamin D-binding protein, to be systemically transported to tissues that express 1α-hydroxylase (CYP27B1) [16]. The second hydroxylation, which generates the bioactive metabolite 1,25-dihydroxyvitamin D3 (calcitriol), occurs at the renal proximal tubular cells that are rich in CYP27B1 [17]. This reaction involves the sequential reduction of flavoprotein, renal ferredoxin, and cytochrome P-450 [18]. A critical physiological role in skeletal homeostasis is mediated by calcitriol. Concisely, hypocalcemia stimulates parathyroid glands to release parathyroid hormone, which activates renal CYP27B1 enzyme function, resulting in calcitriol production. Besides, parathyroid hormone stimulates osteoclast maturation to release calcium and phosphate from the bones. Calcitriol also reduces renal calcium excretion and increases calcium absorption from foods in the intestine. When normal calcium levels are obtained, calcitriol exerts a feedback regulation in the parathyroid gland, downregulating CYP27B1 activity to avoid VitD intoxication [14]. Besides the kidneys, 1α-hydroxylase has been reported in many tissues including bone, placenta, prostate, and parathyroid gland. In addition, several cancer cells and immune cells, such as macrophages, T lymphocytes, and DCs, are also able to produce this enzyme [19,20].

3. Immunomodulatory properties of VitD

First evidences of VitD role in the immune system regulation date from the 80s. Haq [21] demonstrated that active VitD, but not its non-active form, blocked the production of IL (interleukin)-2 and consequently inhibited T-cell proliferation. Based on this downmodulatory effect, the potential of VitD to increase organ survival in experimental allograft transplantation was also evaluated. First studies in this field were based on the *in vitro* immunosuppressive effects of VitD and its analogs. One of the most evident toxic effects of high VitD doses, which are usually required to avoid transplant rejection, is hypercalcemia. To avoid this and other toxic effects such as bone resorption, many efforts were done to develop synthetic structural analogs of active VitD that still preserved its immunomodulatory properties [22]. When tested *in vivo*, a 20-epi-vitamin D3 analog did not prolong renal allograft survival in Lewis rats and also led to the development of hypercalcemia [23]. These authors emphasized the importance of more experimental studies to evaluate the potential of VitD and its analogs to prevent graft rejection. Later, Hullett et al. [24] successfully demonstrated that Lewis rats orally receiving active VitD presented prolonged survival heart allografts without hypercalcemia. Over the years, a much broader role of VitD in the immune system was disclosed and the mechanisms underlying its immunomodulatory effects were progressively elucidated. Currently, calcitriol is largely known to modulate both innate and adaptive immunity through its binding to VDR, which is present in a multitude of immune cells. Although VitD can bind to both genomic and non-genomic targets, the most important immunomodulatory properties are elicited by genomic mechanisms [25].

It is well known that VitD stimulates the innate immune system by enhancing the antimicrobial ability of monocytes and macrophages. This effect is mainly associated with TLRs activation and increased release of cathelicidin and IL-1β by these cells [26]. Clinical evidences suggested a strong correlation between a poor VitD status and an increased susceptibility to infections. VitD has also been linked to more severe infectious diseases [27–29]. Moreover, Nouari et al. [30] recently demonstrated that active VitD can enhance the microbicidal activity of human monocyte-derived macrophages against *Pseudomonas aeruginosa*.

Conversely, VitD has an inhibitory effect on the adaptive immune system. It directly targets APCs, which are a very important link between the innate and adaptive immunity. In this sense, conventional APCs as DCs are profoundly affected by VitD. The mechanisms underlying the effects of VitD on DC function were recently reviewed by Barragan et al. [31]. *In vitro* treatment with active VitD or its analogs inhibits both differentiation and maturation of human and murine DCs leading to changes in its phenotype and function [32]. The immature or semi-mature state induced by VitD is generally characterized by a decreased expression of co-stimulatory molecules such as CD40, CD80, and CD86. This state determines a tolerogenic DC phenotype associated with reduced IL-12 and increased IL-10 production. The addition of VDR agonists or active VitD during differentiation of DCs *in vitro* determines a reduction in subsequent T-cell proliferation and also in interferon-gamma (IFN-γ) production [33]. Tolerogenic DCs are also able to induce the development of Treg cells that are mainly characterized by the expression of CD4 and CD25 molecules and production of anti-inflammatory

cytokines such as IL-10 and transforming growth factor-β (TGF-β) [34]. As mentioned before, Treg cells play a major role in controlling inflammatory immune responses. The main mechanisms underlying their suppressive activity include the induction of inhibitory molecules such as cytotoxic T-lymphocyte antigen 4, the production of inhibitory cytokines that leads to impaired T-cell expansion and the release of granzymes and perforin that trigger T-cell death [35]. Chambers et al. [36] demonstrated that addition of active VitD on human CD4+ T lymphocytes significantly increased the expression of forkhead box protein P3 (Foxp3) that characterizes Treg cells.

The direct effect of VitD on T cells was the first evidence of the immunomodulatory activity of this hormone. Active VitD suppresses Th1 inflammatory immune response through inhibition of IL-2 and IFN-γ production, which are the main cytokines produced by this Th cell subset. This subject was revised by Lemire et al. [37]. These authors described that VitD preferentially inhibited Th1 functions having little effects over Th2 cells. At that time, they already suggested that this vitamin could have a potential therapeutic application in Th1-mediated diseases as is the case of some autoimmune pathologies.

Many inflammatory responses are also related to the development of Th17 cells and its signature cytokine named IL-17. It is largely known that this T-cell subpopulation is involved in the pathogenesis of a variety of inflammatory and autoimmune disorders [38]. In this context, Th17 cell pathogenicity is frequently related to a Th17-Th1 functional plasticity that is regulated by the cytokine milieu [39]. The immunomodulatory effects of VitD on Th17 cells are not clear and depend upon the disease. Most of what is known concerning VitD effect on these cells is based on experimental studies. For example, oral treatment with active VitD prevented and partly reversed experimental autoimmune uveitis in mice. This effect was related to both decreased IL-17 production and impaired development of Th17 cells [40]. Moreover, Chang et al. [41] demonstrated that active VitD treatment protected mice from experimental autoimmune encephalomyelitis (EAE) by inhibiting the differentiation and further migration of Th17 cells to the central nervous system (CNS). Even though the effect of VitD on animal models is evident, human data are controversial and there is not a consensus in the literature yet.

Data on the effects of VitD on the development of Th2 cells are also conflicting. This T-cell subset is able to suppress Th1 inflammatory immune response through the production of anti-inflammatory cytokines such as IL-4 and IL-5. A direct effect of active VitD on Th2 cells was demonstrated by Boonstra et al. [42]. Even in the absence of APCs, these authors observed an increased frequency of IL-4-, IL-5-, and IL-10-producing murine CD4+ T cells after *in vitro* stimulation with VitD. In addition, there was a decrease in the frequency of IFN-γ-producing cells. However, Staeva-Vieira and Freedman [43] demonstrated that active VitD inhibited the *in vitro* production of both, IFN-γ and IL-4 by murine CD4+ T cells.

Other T-cell subsets such as CD8+ T cells and natural-killer T cells (NKT) are also targets of VitD. Chen et al. [44] demonstrated that active VitD signaling through VDR is essential to control pathogenic CD8+ T cells in inflammatory bowel diseases. The importance of VDR was also highlighted by Yu et al. [45] who demonstrated a critical role of VDR expression in the development of induced NKT cells from mice fed with synthetic diets containing active VitD.

There are few studies concerning the impact of VitD on B cells. *In vitro* assays indicated that the active form of VitD inhibited the production of immunoglobulin E and increased IL-10 production by B cells [46,47]. Similarly to the effect over DCs, active VitD also downregulated the expression of co-stimulatory molecules at the surface of human B cells. Drozdenko et al. [48] demonstrated that the antigen-presenting function of B cells was compromised by *in vitro* addition of active VitD to B and T cell co-cultures. The authors detected a reduced expression of the co-stimulatory molecule CD86 in B cells along with diminished T-cell expansion and lower cytokine production by these cells. A general scheme indicating some of the most relevant effects of VitD on innate and adaptive immunity is displayed in **Figure 1**.
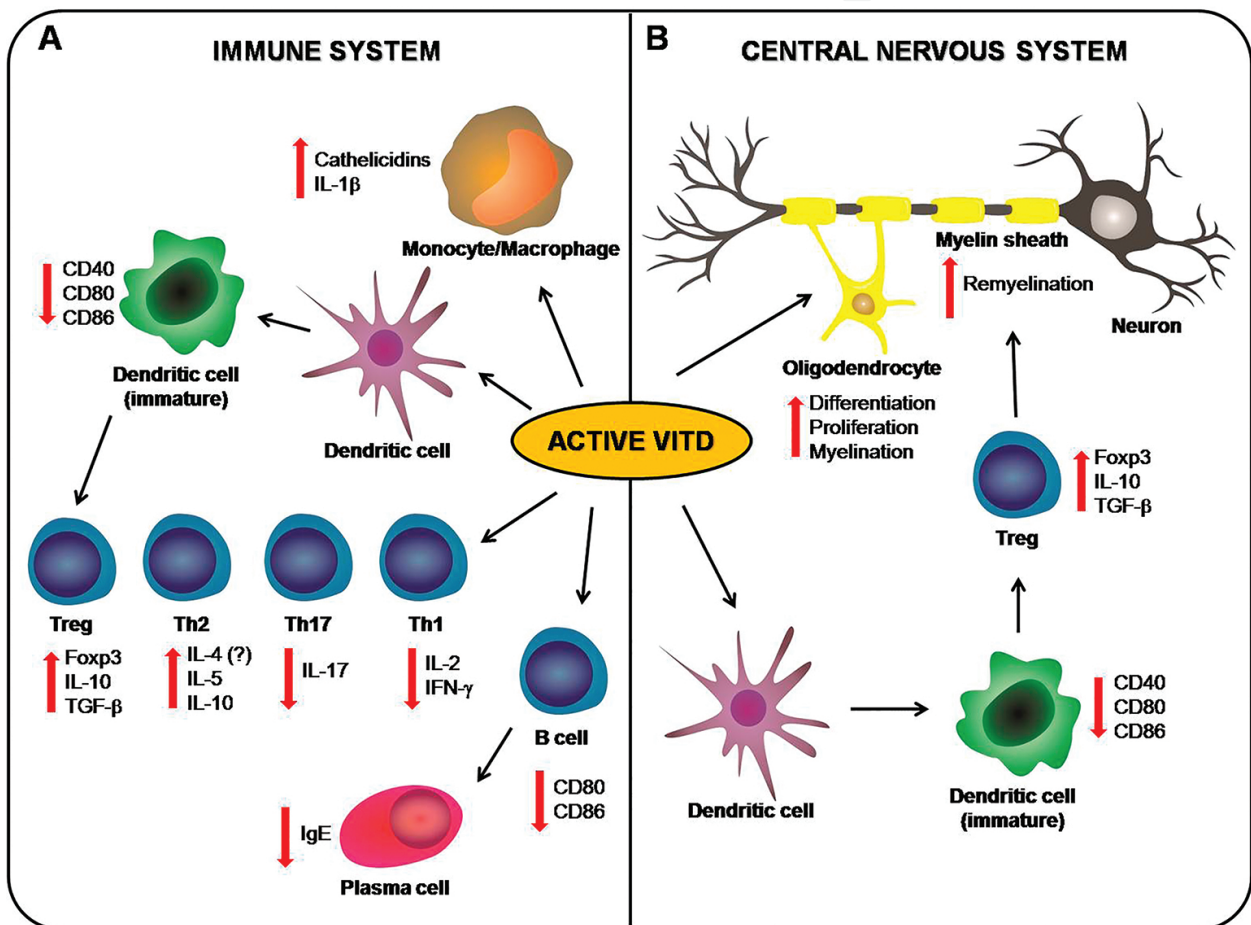
Figure 1. VitD action on the immune and the central nervous systems. (A) Effect of active VitD on the innate and the adaptive immunity cells and (B) direct and indirect effects of active VitD on the central nervous system.

The immunomodulatory potential of VitD has been widely explored in the field of autoimmune diseases. Epidemiological studies demonstrated that low VitD is correlated with a higher incidence of autoimmune diseases. Besides, genetic factors as VDR polymorphisms are also linked to autoimmune disorder susceptibility. The association between VitD and systemic and organ-specific autoimmune diseases, including multiple sclerosis (MS), was carefully reviewed by Agmon-Levin et al. [49].

4. Epidemiological evidence that VitD is relevant in MS

MS is an autoimmune disease characterized by the activation of self-reactive T cells specific for CNS antigens. This immune response triggers an initial inflammation in brain and spinal cord that is then followed by demyelination, axonal damage, and scar formation [50]. The pathogenic immune response observed in MS is mainly mediated by Th1 and Th17 [51]. About 85% of MS patients present with a biphasic disease characterized by alternating episodes of neurological disability and recovery, which is entitled as relapsing remitting MS (RRMS). Within 20–25 years, 60–70% of these patients progress to a secondary-progressive disease that is characterized by progressive neurological deterioration. Approximately 10% of the patients display a disease course classified as primary progressive MS, which is characterized by a continuous decline in neurological performance without any recovery episode [52]. Magnetic resonance imaging (MRI) is playing a prominent role in the diagnosis and also in the analysis of MS therapy efficacy [53]. As mentioned before, autoimmune diseases result from the interactions of environmental and genetic risk factors. Environmental risk factors considered essential for MS development include infections and non-infectious factors that comprise differences in diet and other behaviors, such as cigarette smoking and sunlight exposure [54,55]. The development of MS has been strongly associated with viral and bacterial infections [54,56]. More recently, a possible relationship between MS and Candida species was proposed [57–59]. Our research team recently demonstrated that previous infection with *Candida albicans*, a commensal and opportunistic human pathogen, aggravates the clinical signs of EAE [60].

Epidemiological data on MS incidence and prevalence drew attention to a possible link between the geographical distribution of the disease and exposure to the sun, UV radiation/intensity, and VitD levels. This sunshine hypothesis also known as latitude-gradient effect was initially proposed by Limburg [61] that suggested a correlation between higher MS occurrence and increasing distance from the equator. According to the World Health Organization [62], the highest prevalence of MS occurs in Europe (80 per 100,000 people) and the lowest prevalence in Africa (0.3 per 100,000). More recently it was reported that, until 2013, the number of MS was higher in northern hemisphere and lower in southern hemisphere, with the exception of Australia and New Zealand [63]. A latitudinal variation was also identified in the continents. For example, geospatial analysis carried out in North American regions showed an inverse correlation between MS and UV radiation, that is, higher MS rates have been associated with lower UV radiation due to a south-north latitudinal gradient [64]. Interestingly, a series of lifestyle changes that include sun evasion associated with skin protection and extra time indoors, or increased charter tourism to warmer countries during the winter, seems to abolish latitude effects on UV radiation exposure [65]. According to these authors, this association between sun exposure and MS can be determined by distinct effects: by the VitD generated by sun exposure, by direct sun effects, or by a combination of both. These possibilities are reinforced by data from experimental animals and also from dietary studies in human populations. Dermal application of VitD ointments and UV radiation in VDR knockout mice were both able to induce Treg cells [66]. Further study indicated that these UV-induced Treg

cells were able to migrate to the CNS of mice with EAE where they downregulated the inflammatory activity [67].

A lower prevalence of MS in some northern countries, which in a general way are expected to have a higher number of patients with the disease, could be explained by VitD-related dietary factors. For example, VitD sufficiency could be achieved through a traditional diet that includes fatty fish and cod liver oil. This possibility has been suggested to explain the reduced risk of MS in Norway that is located at the north of the Arctic Circle [68]. The relevant role of dietary VitD intake in MS was examined in two large cohorts of women: the Nurses' Health Study (NHS; 92,253 women followed between 1980 and 2000) and the Nurses' Health Study II (NHS II; 95,310 women followed between 1991 and 2001). The authors concluded that intake of VitD from supplements had a protective effect on the risk of developing MS [69]. A recent study with 953 MS patients indicated an inverse association between MS risk and the dose of cod liver oil during adolescence, suggesting that this stage of life is an important susceptible period for adult-onset MS, reinforcing the importance of dietary VitD as a risk factor for MS [70]. Altogether these data supported the possibility that MS patients could have lower levels of VitD. Regarding this, the largest study to date compared VitD levels present in Iranian MS patients (n = 700) to the ones found in healthy individuals (n = 1000) and demonstrated that VitD levels were significantly lower in patients with MS [71]. Strong evidences also support the likelihood that low VitD levels can be related to disability and progression of this disease. In a study with 267 patients, lower serum VitD levels were also associated with higher rates of both relapse and disability [72]. Other authors showed an association between a low VitD status at the start of RRMS and the early conversion to secondary progressive MS [73]. The possible effect of VitD levels in the therapeutic efficacy of interferon beta 1b(IFN-β-1b), fingolimod (FTY), and glatiramer acetate (GA) was also investigated. Among patients treated with IFN-β-1b, higher VitD levels were associated with a reduced risk of relapse [74], whereas lower VitD levels early in the disease course correlated with a strong risk factor for long-term MS activity and progression [75]. In a similar way, in FTY-treated patients, higher VitD levels were associated with an approximately 50% reduction in new inflammatory events and in relapses [76]. By contrast, there was no significant benefit of higher VitD levels with respect to inflammatory events, relapses, or disability progression in GA-treated patients [76]. The strong correlation between low VitD levels and higher MS susceptibility reinforces the hypothesis that VitD deficiency leads to MS and/or disease progression and stimulates new researches focused on supplementation of these patients with VitD.

5. Supplementation of MS patients with VitD

The recent identification of VitD as a risk factor for MS susceptibility, and more recently as a potential modifier of disease course, inspired several clinical trials in relapsing MS [77]. It has been proposed that VitD supplementation is a low-cost and a low-risk intervention that may potentiate the efficacy of certain treatments against MS, without the risk of provoking serious adverse events as occurs with other combination therapies [76]. In effect, many patients are being already supplemented with VitD. However, it is not known whether supplementation has a significant impact on MS progression. A clinical trial (NCTO1339676) employing oral

supplementation with active VitD (20,000 IU/week, cholecalciferol, Dekristol) administered once a week during 12 months together with IFN-β-1b resulted in reduction of MRI lesions in the brain of MS patients [78]. In another clinical trial (NCT 00785473), this same dose (20,000 IU/week, cholecalciferol, Dekristol) was administered during 24 months in RRMS patients under treatment with IFN-β-1b, GA, or natalizumab. Even though the patients presented a significant increase in serum VitD levels, the markers of systemic inflammation were not modified. The authors suggested that the anti-inflammatory effects of VitD supplementation are limited to RRMS patients with VitD insufficiency or to earlier stages of the disease [79]. A higher dose of VitD3 (50,000 IU/week) administered by oral route during a short period (2 months) reduced disability in RRMS patients and surprisingly upregulated IL-6 and IL-17 gene expression in the peripheral blood mononuclear cells of these patients [80]. Similarly, the same VitD dose (50,000 IU) administered by oral route every five days for 3 months in 94 RRMS patients under treatment with IFN-β-1b reduced disability of these patients but also increased IL-17 serum levels in comparison to a placebo group [81]. Investigations in this area suggested that changes in IL-17 levels could be related to the adopted VitD doses. For example, Golan et al. [82] demonstrated that IL-17 serum levels were significantly increased in a lower dose group (800 IU/per day), whereas patients that were taking higher doses (4370 IU/per day) presented heterogeneous IL-17 responses: 40% of them had decreased serum IL-17 levels, whereas 45% had increased IL-17 levels after three months of supplementation. These authors suggested that IL-17 data must be interpreted with caution as serum IL-17 is not an established biomarker of MS disease activity. Furthermore, IL-17 serum levels before treatment with IFN-β could not be correlated to disease activity parameters [83]; IL-17 also showed a trend toward higher levels in MS patients with inactive disease compared to those with active disease [84]. More recently, 40 patients with RRMS were randomized to receive 10,400 IU or 800 IU of cholecalciferol daily for 6 months. Mean increase of VitD levels from baseline to the ones detected at final visit was larger in the high-dose group than in the low-dose one and adverse events were minor and did not differ between the two groups. Interestingly, in the high-dose group, but not in the low-dose one, there was a reduction in the proportion of IL-17+CD4+ T cells. The authors concluded that daily cholecalciferol supplementation with 10,400 IU is safe and well tolerated in patients with MS and determines *in vivo* pleiotropic immunomodulatory effects [85]. Considering that IL-17 is an important cytokine involved in MS pathogenesis, further studies are needed to clarify the role of VitD on these unexpected elevated IL-17 levels. Therefore, until nowadays it is not possible to consider IL-17 as a biological marker for VitD levels in human body.

The researches done so far strongly suggest that VitD supplementation could be useful in MS treatment. However, the exact doses to be prescribed to patients presenting different clinical symptoms are still waiting to be determined [86]. Regarding the side effects of VitD that include hypercalcemia [87] and the imbalance in serum concentration of parathyroid hormone [88], monitoring serum VitD would also be extremely important. In spite of the findings that VitD directly regulates the nervous system development and function [89], there is no scientific evidence to support its use as a monotherapy for MS in clinical practice [90]. Recent human trials concerning VitD supplementation in MS patients suggest that higher VitD doses are more efficient to control the symptoms and disease inflammatory markers. Nonetheless, to fix the

ideal dose, it is essential to measure VitD serum levels before supplementation and to follow up the patients by constantly monitoring side effects. It is important, however, to highlight that the ideal dose could vary from one patient to another. The possible use of VitD analogs devoid of side effects must be also evaluated. World Health Organization (WHO) and Multiple Sclerosis International Federation (MSIF) published in 2008 the first Atlas of MS [62], correlating the epidemiology, diagnosis, and therapy. To the best of our knowledge, WHO did not define a specific VitD dose to treat MS.

6. Therapeutic effect of VitD in EAE

Experimental autoimmune encephalomyelitis (EAE) is an animal model universally employed to investigate mechanisms of inflammation in the CNS in the context of MS. EAE is mainly induced in rodents either by active immunization with CNS antigens associated with adjuvant or by passive transfer of CNS-specific T cells. Most of the therapeutic procedures adopted nowadays were initially tested in murine EAE [91]. In 1991, it was demonstrated that VitD administration every other day for 15 days, starting 3 days before EAE induction, significantly prevented disease development and prolonged the survival of SJL/J mice [92]. This was the first report concerning the therapeutic potential of VitD on EAE. To avoid undesirable hypercalcemia *in vivo*, the immunomodulatory activity of VitD analogs were confirmed and they were equally efficient to suppress EAE development [93,94]. Since then, EAE has been widely employed to understand the mechanisms involved in VitD efficacy against MS. In this regard, one of the first studies was done with the Lewis rat model. The authors observed that VitD administered after the beginning of clinical signs determined significant clinical improvement. This therapeutic effect was associated with a striking decrease in the number of CD4+ cells, macrophages, and activated microglia in the CNS [95]. VDR is also essential for the beneficial effects of VitD on EAE since VitD treatment was not able to prevent disease manifestations in VDR-knockout mice [96]. The efficacy of VitD over EAE has also been attributed to effects on cells from the innate immunity. It decreases macrophage accumulation [97], inhibits chemokine synthesis and inducible NOS, and also suppresses CD11b+ monocyte recruitment into the CNS [98]. NKT cells also contribute to the protective effect of VitD on murine EAE. All mice lacking NKT cells [CD1d(−/−)] presented EAE symptomatology upon VitD administration, whereas the same treatment completely avoided EAE development in wild-type mice [99]. More recent data revealed that VitD administration induces tolerogenic DCs in the lymph nodes, which leads to suppression of encephalitogenic T cells, resulting in less inflammatory response in the CNS [100].

Critical effects of VitD on CD4+ T cells have been reported, whereas it is not evident if this vitamin affects CD8+ T cells, which express the highest concentrations of VDR. The effect of VitD on CD8+ T cells in EAE was evaluated in one report. The authors demonstrated that VitD inhibits EAE development even in mice lacking functional CD8+ cells, suggesting that they were not essential for VitD-suppressive effect in murine EAE [101]. The conception that the CD4+ T-cell subset was the main VitD target during EAE therapy was then established. VitD treatment triggered a reduction in the total number of lymphocytes, while the amount of IL-4

and TGF-β-1 transcripts increased in the CNS of EAE mice [102]. Still regarding anti-inflammatory cytokines, VitD therapy was reported to be much less effective in preventing EAE symptoms in IL-4-deficient mice [103] and also failed to inhibit EAE in mice with a disrupted IL-10 or IL-10R gene [104]. A more recently described profile of CD4+ T cells termed Th17 plays a critical role in numerous inflammatory conditions and autoimmune diseases. In this context, researchers showed that VitD can inhibit the differentiation and migration of Th17 cells to the CNS, ameliorating EAE symptoms [41,105].

After the first demonstration that VitD leads to induction of CD4+CD25+Foxp3+ cells with suppressive activity *in vitro* [106] and that these regulatory cells are directly involved in the natural resolution of EAE [107], many studies validated the correlation between VitD treatment and the increment of a Foxp3+ regulatory profile in EAE [99,103,104] (**Figure 1B**). The potential for reversing inflammatory and demyelinating processes in the CNS has been attributed to an augmented generation of Foxp3+ Treg cells in the periphery and their further migration to the CNS [100,108]. New therapeutic approaches have also been tested to improve VitD efficacy in EAE. A synergistic effect was found by association of VitD with estrogen, which determined more CD4+Helios+Foxp3+ Treg cells and fewer CD4+ T cells among CNS mononuclear cells, preventing EAE development [109]. In addition to the large contribution of VitD immunomodulatory activity in EAE, this treatment can also directly act on neural cells promoting CNS remyelination and other neuroprotective effects (**Figure 1B**). *In vitro* assays indicated that this vitamin significantly enhanced proliferation of neural stem cells and their differentiation into neurons and oligodendrocytes [110]. In addition, VitD treatment modulated autophagic activity and neuroapoptosis in EAE mice. As autophagy is an evolutionarily conserved cellular catabolic process that recycles damaged organelles and its inhibition causes neurodegeneration in mature neurons, this process plays an essential role in maintaining neuronal homeostasis [111]. In summary, VitD controls EAE symptoms through reduction of inflammatory immune response and elicitation of a regulatory profile. As EAE reproduces specific features of the histopathology and neurobiology of MS [112], highlighting these mechanisms in rodent models is essential to translate VitD supplementation to MS patients.

Emphasis has been given to specific therapies, that is, to procedures that target CNS antigen and that would be, therefore, more efficient and devoid of side effects. In this context, MOG administration by different routes as intravenous [113], oral [114] or nasal [115], was able to suppress EAE symptoms. Various formulations containing myelin antigens were tested to control EAE. MOG conjugated with nanoparticles [116], mannan, [117] or inserted into a plasmid DNA [118] reduced EAE symptoms through induction of Foxp3+ Treg cells and dowmodulation of Th17 and Th1 cells. Our research group has been working in this context. Considering that an antigen from the CNS can provide the required specificity and that VitD is endowed with a strong downmodulatory potential, we anticipated that VitD could work as a tolerogenic adjuvant. Differently from the conventional immunogenic adjuvants that reinforce the immune response, the denominated tolerogenic adjuvants have the ability to downmodulate or modify the specific immune response when associated with specific antigens. Confirming this hypothesis, we recently demonstrated that a combined therapy with MOG + VitD blocked EAE development. This elevated efficacy was correlated

with reduced production of IL-6 and IL-17 by spleen and CNS cell cultures stimulated with MOG, reduced splenic DC maturation, and also a striking decline in CNS inflammation [119] (**Figure 2**).
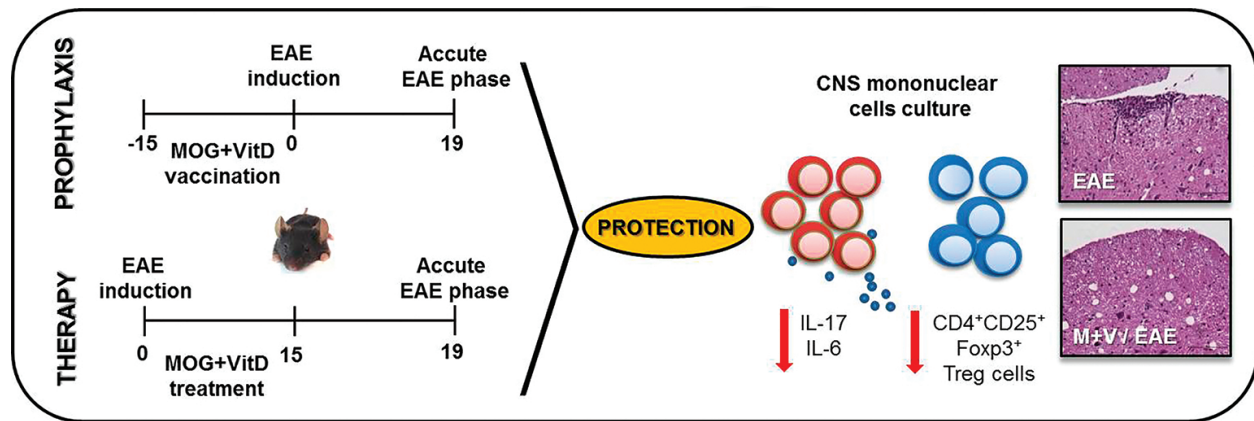
Figure 2. MOG + active vitamin D3 association strategy for EAE prophylaxis and treatment. C57BL/6 mice were vaccinated or treated with this association and the effect on EAE was evaluated in the acute EAE phase. Both strategies decreased production of inflammatory cytokines by CNS mononuclear cells, frequency of CD4+CD25+Foxp3+ Treg cells, and inflammation in the CNS.

7. Prophylactic effect of VitD on EAE

Prophylactic strategies in EAE, and also in other autoimmune pathologies, are based in the concept of "inverse vaccination." This procedure refers to the use of an immunization protocol that, differently from classical vaccination, aims to achieve an antigen-specific tolerogenic state [120]. Even though the term "inverse vaccination" could also be used as a therapeutical strategy, in this text we applied it only in the context of prophylactic vaccination. The majority of the prophylactic strategies in EAE have been done by administration of a diversity of MOG formulations delivered by distinct routes. A few examples of these procedures and the main histological and immunological findings are illustrated in **Table 1**.

The prophylactic potential of VitD (or analogs) alone or associated with other pharmaceuticals has been tested in EAE. The adopted experimental protocols are not standardized and therefore, different amounts of VitD are administered by distinct routes. Time periods chosen for VitD administration in relation to EAE induction are also variable and some procedures consist in prolonged administration periods, even reaching the disease clinical phase. However, a general consensus is that VitD is able to improve clinical disease manifestation and also to trigger evident effects on the CNS and the immune system. Some of the effects observed in mice with EAE that were previously injected with VitD are exemplified in **Table 2**.

Peptide formulation	Animal model	Effects	References
Plasmid DNA vaccines encoding MOG35–55	C57BL6/J mice	↓Microglia/macrophage activation, astrogliosis, and axonal damage ↑CD4+CD25+Foxp3+ Treg	Fissolo et al. [118]
MOG35–55 conjugated to mannan, intradermally	C57BL/6 and SJL/J mice	↓Demyelination ↓Inflammatory infiltrates	Tseveleki et al. [117]
Tolerogenic DC pulsed with MOG40–55	C57BL/6 mice	↑IL-10 production by MOG-stimulated splenocytes ↑CD3+CD4+CD25+FoxP3+ cells	Mansilla et al. [121]
MOG35–55-PLGA + IL-10-PLGA, subcutaneously	C57Bl/6 mice	↓IL-17 and IFN-α production by splenocytes↓Demyelination score	Cappellano et al. [122]

Table 1. MOG prophylactic procedures in EAE.

Route	Animal model	Effects	References
Diet	CD8+ $^{-/-}$ mice	Protection independent of TCD8+ cells	Meehan and DeLuca [101]
Intraperitoneally	C57BL/6 mice	↓MyD88, IRF-4, IRF-7 and NF-kB expression ↓Several TLRs	Li et al. [123]
Oral, gavage	C57BL/6 mice	Intact blood–CNS barrier ↓Inflammatory infiltrates in the CNS	Grishkan et al. [124]
Intraperitoneally	C57BL/6 mice	↓Demyelination ↑Beclin-1 expression in neurons	Zhen et al. [111]

Table 2. Vitamin D3 prophylactic procedures in EAE.

The combination of VitD with other substances as calcitonin [125], IFN-β [126], bisphosphonate [127], rapamycin [128], and cyclosporine [129] has determined cooperative effects over EAE control. We recently tested the association of VitD with MOG as a prophylactic approach to control EAE development. Again, in this procedure, we explored the concept of VitD as a tolerogenic adjuvant. This concept and its potential application to trigger self-tolerance in autoimmune diseases were conceived by Kang et al. [130]. These authors validated this hypothesis by demonstrating that FK506 (tacrolimus) associated with MOG was prophylactic in encephalomyelitis [131]. In this context, we hypothesized that active VitD could also behave as a tolerogenic adjuvant if associated with a CNS-specific antigen. Vaccination with MOG associated with VitD, before EAE induction in C57BL/6 female mice, determined a significant clinical improvement characterized by absence of clinical score and no body weight loss. An impressive reduction in CNS inflammation, DC maturation and also cytokine production by CNS and spleen cell cultures was detected in these vaccinated animals [132]. As described in Section 6 of this chapter, this combination of MOG with VitD was also very efficient as a therapeutic procedure in the EAE model. This prophylactic and therapeutic potential of the MOG/VitD association in EAE is illustrated in **Figure 2**. The possible use of VitD as a tolero-

genic adjuvant in association with other self-antigens, as a strategy to control autoimmune pathologies, warrants future investigation. In our opinion, the fact that VitD is already accepted for human supplementation will facilitate its adoption for MS treatments based on its association with neuronal self-antigens.

Acknowledgments

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