CNS Neuroscience & Therapeutics

ORIGINAL ARTICLE

Vitamin D_3 Induces IDO$^+$ Tolerogenic DCs and Enhances Treg, Reducing the Severity of EAE

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Keywords
Autoimmunity; DCs; T-cell activation; Tregs; Vitamin D_3.

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Received 8 November 2012; revision 16 January 2013; accepted 17 January 2013.

doi: 10.1111/cns.12071

SUMMARY

Background: A growing body of evidence supports the hypothesis that vitamin D is an important environmental factor in the etiology of T-cell-mediated autoimmune diseases such as multiple sclerosis (MS). **Aim:** The purpose of this study was exploring the mechanisms underlying the beneficial effect of vitamin D3 in encephalomyelitis (EAE). **Methods:** We treated monophasic experimental autoimmune EAE, induced in Lewis rat, with vitamin D3 and adoptively transfer tolerogenic bone marrow-derived DCs generated in the presence of vitamin D3. **Results:** This study provides evidence that the *in vivo* administration of vitamin D_3, as well as the adoptive transfer of vitamin D_3-induced IDO$^+$ immature/tolerogenic dendritic cells, leads to a significant increase in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in the lymph nodes in a rat model of MS, experimental autoimmune EAE. Concomitant with the increase in this cell population, there is a significant decrease in the number of autoreactive T cells in the central nervous system. Bone marrow-derived DCs cultivated in the presence of vitamin D_3 present a tolerogenic profile with high IL-10, TNFα, and IDO expression and decreased MHC-II and CD80 expression. The adoptive transfer of IDO $^+$ DCs induces a significant increase in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells in the lymph nodes, comparable with vitamin D_3 treatment. **Conclusion:** These mechanisms contribute actively to the generation of a microenvironment in the lymph nodes that suppresses the activation of encephalitogenic T cells, resulting in the downregulation of the inflammatory response in the central nervous system.

Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) in humans [1]. Although the etiology of MS is unknown, it is widely accepted that the disease results from complicated interactions between multiple genes and the environment. The increasing prevalence of the disease with increasing latitude suggests a strong link between low exposure to sunlight and a high risk of MS [2,3]. This may be explained, at least in part, by a vitamin D_3 deficiency in patients with MS [2], which can be caused by low vitamin intake and/or by limited vitamin D_3 synthesis in the skin, particularly in climates that are not conductive to outdoor activities [3].

Previous studies provide evidence for the beneficial effects of vitamin D_3 treatment in experimental autoimmune encephalomyelitis (EAE), an experimental model of MS [4–6]. The cytokines that are produced by Th1 and Th17 CD4 T lymphocytes are linked directly to the pathology of the disease, especially IFNγ, IL17, and TNFα, whereas Th2-/Th3-produced cytokines, such as IL-10 and TGFβ_1, ameliorate EAE [7–13]. Previous studies have demonstrated that treatment with vitamin D_3 or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) can inhibit the IL-12/IFNγ axis [14] as well as Th17 differentiation [15].

Despite the direct effect of vitamin D_3 on T cells, many studies have also described a crucial role for DCs in the immunomodulation promoted by vitamin D_3 [16,17]. DCs constitutively express the vitamin D receptor (VDR) and are able to perform the conversion of vitamin D_3 into its active form [18]. The activation of VDR by $1,25(OH)_2D_3$ stimulates the tolerogenic activity of dendritic cells by acting in the differentiation and maturation of these cells [19,20]. Moreover, tolerogenic dendritic cells, which may express STAT3 and IDO, enhance the activity of regulatory CD4$^+$ and CD8$^+$ regulatory T cells [21].

Regulatory cells that express the transcription factor Foxp3 have a crucial function in activating immune suppression and maintaining immune homeostasis [22], although other regulatory T cells, such as Th3 and T regulatory type 1 (Tr1) cells, also contribute substantially to the active suppression of the autoimmune

response [23–25]. A deficiency in either number or function of Foxp3-positive T cells has been described in both MS and the EAE model [26,27].

In vitro studies have demonstrated that DCs cultivated in the presence of vitamin D_3 are able to convert naïve T cells into Foxp3 regulatory T cells or Tr1 cells [28–31]. However, no observations about the *in vivo* effects of vitamin D_3 on $CD4^+CD25^+Foxp3^+$ regulatory T cells in the EAE model have been published.

This study was designed to investigate the effects of both treatment with vitamin D_3 and the induction of tolerogenic activity of dendritic cells in the generation of Foxp3 regulatory T cells in the EAE model.

Materials and Methods

Animals

Six- to eight-week-old female Lewis rats were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and established as a colony at the University of Campinas Breeding Center, where they were housed and maintained under pathogen-free conditions in the university animal facility. The experimental animals were allowed access to standard rodent chow and water ad libitum, with temperature maintained between 21° and 23°C and a 12-h light/12-h dark cycle. The animals were age matched for individual experiments and randomly distributed into treatment or control groups. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care and approved by the University Committee for Ethical Animal Experimentation (CEEA/UNICAMP #2038-1).

Antigens and EAE Induction

Each animal received a subcutaneous injection of 50 μg gpMBP, purified from guinea pig brain, or 15 μg of $gpMBP_{73-86}$ peptide (QKSQRSQDENPV), emulsified in complete Freund's adjuvant containing 2 mg/mL of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). The clinical expression of the disease was graded on a clinical index scale 0–5 in accordance with previous work [32].

Vitamin D_3 Treatment

Vitamin D_3, (cholecalciferol (D3); Sigma Chem., MO, USA) was diluted in 80% polyethyleneglycol and given intraperitoneally (i.p.) or orally, beginning on day 0 of EAE induction and continuing until day 20 after immunization. Six different doses were used, 2 i.p. (10 and 15 μg/Kg/day) and 4 oral (2.5, 5, 10 and 15 μg/Kg/day). The control group was fed or injected with vehicle alone. Oral administration (feeding) was performed with a gavage needle (200 μL of final volume) (*15 animals per group in three independent experiments*).

Quantification of MBP Antibodies

Briefly, 96-well microtiter plates (NUNC–Denmark) were coated with 25 μg/mL of MBP in 0.1MNaHCO3 (pH = 8.5) and left overnight at 4°C. Following blocking with 3% bovine serum albumin in PBS for 2 h at room temperature, serum was added and incubated overnight at 4°C. Then, 1.0 μg/mL of detection antibody for mouse total IgG or anti-isotype IgG1 (Sigma Chem. MO, USA) was added, followed by peroxidase substrate. Optical density (OD) was determined at 492 nm. To avoid variation in results, all serum samples were tested at the same time.

Lymphocyte Proliferative Response

Lymph node cells were removed at 12 days postimmunization, pooled, and mechanically dispersed through a nylon mesh to isolate single-cell suspensions. The cells in suspension were washed twice in Hanks solution and resuspended in RPMI 1640 with 2-mercaptoethanol and 5% heat-inactivated fetal bovine serum (Sigma Chemical Co. St. Louis, MO, USA) prior to stimulation with 10 μg/mL of $gpMBP_{73-86}$ for 96 h. The incorporation of ^{3}H-thymidine was assessed by standard liquid scintillation techniques (*five independent experiments*).

Histology

Ten-micrometer sections were cut from snap-frozen spinal cords of the rats of three groups (naïve, untreated, and vitamin D_3 treated) at the peak of EAE; the sections were fixed with 4% formaldehyde and stained with hematoxylin and eosin (H&E) (*20* slides per group from *five independent experiments*).

Antibodies and Flow Cytometer Analysis

All analyses were performed in a flow cytometer (FACS canto or FACS Calibur) (BD Bioscience, San. Jose, CA, USA) using FACSDiva, Cell Quest or MDI2.8 software. For Foxp3 labeling, permeabilization buffer (PBS 10% rat serum and 1% Triton) was used. For quantification of $CD4^+$ cells present in the CNS, a known number of PE-beads (BD Bioscience, San Jose, CA, USA) were used. The antibodies used were as follows: anti-11b PE, anti-CD80 PE, anti-MHC-I FITC, anti-MHC-II FITC, anti-Anti-TCR$\alpha\beta$, anti-CD11c, (Serotec), anti-CD4 FITC, anti-CD25 PE, and anti-OX40 (BD Bioscience, San Jose, CA, USA), and anti-Foxp3 APC (eBioscience, San Diego, CA, USA) (each flow cytometer plots are representative from, at least, *five independent experiments*).

Quantitative PCR

mRNA was extracted using Trizol and reverse transcribed to cDNA. TaqMan analysis was performed using a TaqMan ABI Prism 7500 Sequence Detector (PE Applied Biosystems, Darmstadt, Germany). The primers for β-actin, IFNγ, IL-12p40, IL-10, TGFβ_1, TNFα, IDO, CD80, and STAT3 were obtained from Applied Bioscience. The expression of each specific mRNA was normalized to that of a housekeeping gene (β-actin). The data were obtained by independent duplicate measurements. The threshold cycle value of the individual measurements did not exceed 0.5 amplification cycles. For quantitative PCR, DCs were enriched (98%) for CD11-positive cells by sorting using FACSAria (BD Bioscience, San Jose, CA, USA) (each quantitative PCR is resulted from, at least, *five independent experiments*).

DC Generation and Transfer

DCs were generated from bone marrow precursor cells extracted from the tibias and femurs of naïve Lewis rats. Red blood cells were lysed in an NH_4Cl solution, and the cells were cultured in RPMI plus 10% FCS, 50 μmol/L 2-mercaptoethanol, 50 μg/mL gentamicin, and 10 ng/mL GM-CSF. On days 2, 4, 6, and 8, 1 nmol/L vitamin D_3 was added. After 12 days, most of the cells remained adherent and were trypsinized for subsequent analysis and experiments. Animals received an injection of 5×10^5 DCs in 200 μL of PBS into the foot pad 1 day before active EAE induction (*12 animals per group in three independent experiments*).

Statistical Analysis

The statistical significance of the results was determined using a nonparametric analysis of variance (Kruskal–Wallis test) and a Mann–Whitney test (*U*-test). A *P* value smaller than 0.05 was considered significant.

Results

Vitamin D_3 Treatment Reduces the Severity of EAE

Encephalomyelitis was actively induced in Lewis rats by immunization with MBP_{73-86} emulsified in CFA. Vitamin D_3 was administered either orally or i.p. However, no effect on the clinical evolution of EAE was observed when the i.p. route was used (Figure S1 A). Different concentrations of vitamin D_3 were tested orally; doses of 10 or 15 μg/Kg/day administered daily both significantly reduced the severity of the EAE ($P < 0.01$) relative to the untreated group (Figure 1A). The dose of 15 μg/Kg/day was established as a standard dosage for all experiments. These results

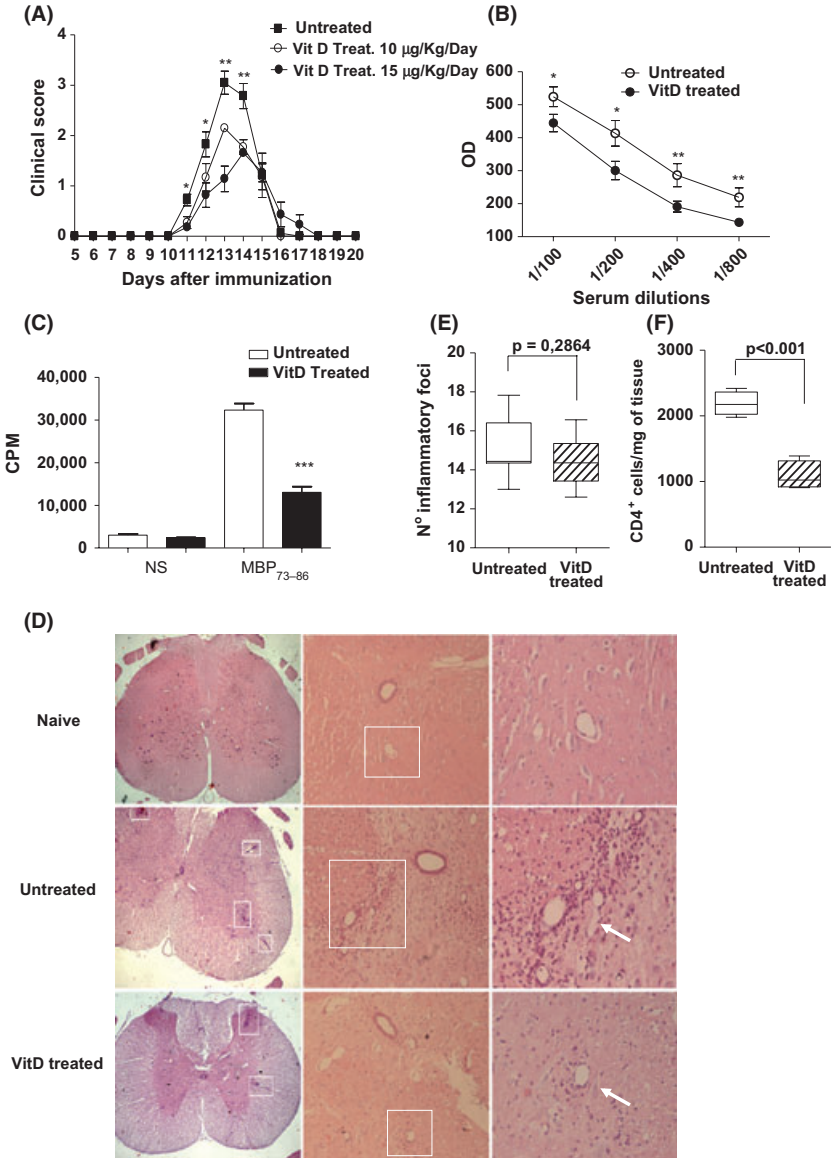

Figure 1 Peripheral and SNC modification of immune response in vitamin D_3-treated animals. The oral treatment with vitamin D_3 10 μg/Kg/Day (white circle) and 15 μg/Kg/Day (black circle) by gavage. The treatment clearly ameliorates the severity of the clinical signs in relation to control encephalomyelitis (EAE) group (black square), during the exacerbation (11–14 d.a.i.) phase of EAE (**A**). Treatment with vitamin D_3 decreases the release of anti-MBP antibodies and diminishes the specific proliferative response of T cells (**B** and **C**, respectively). Histological sections of the spinal cord of a naïve rat, rats from the untreated and vitamin D_3-treated groups (12 days after immunization) stained with H&E (*arrows* indicate inflammatory foci). Transverse sections imaged using an 8x (left column), 20x (center column), or 40x objective lens (right column) (**D**). However, this treatment does not protect the rats from BBB disruption because there are no significant differences in the number of inflammatory foci in the two groups (**E**). The fewer number of $CD3^+CD4^+$ cells was confirmed by flow cytometer quantification of recovered cells from spinal cord after Percoll gradient. The treated animals clearly show a significant reduction in $CD4^+$ cells into the spinal cord tissue in relation to untreated animals (**F**). ** $P < 0.05$, **$P < 0.01$, ***$P < 0.001$*

confirmed previous studies indicating the beneficial effects of vitamin D_3 in the EAE model.

Suppression of Peripheral Immune Response with Vitamin D_3 Administration

The development of EAE is characterized by autoreactive T-cell activation, followed by the migration of these cells into the CNS. The activation of autoreactive T cells takes place in the peripheral lymph nodes, starting after immunization with the neuroantigen. Therefore, the effect of vitamin D_3 was evaluated in the peripheral lymph nodes 12 days after immunization (d.a.i.) with the neuroantigen.

Both antibody production and the proliferative response of the lymphocytes to the MBP antigen were evaluated the vitamin D_3-treated and untreated groups. The level of antibodies against MBP

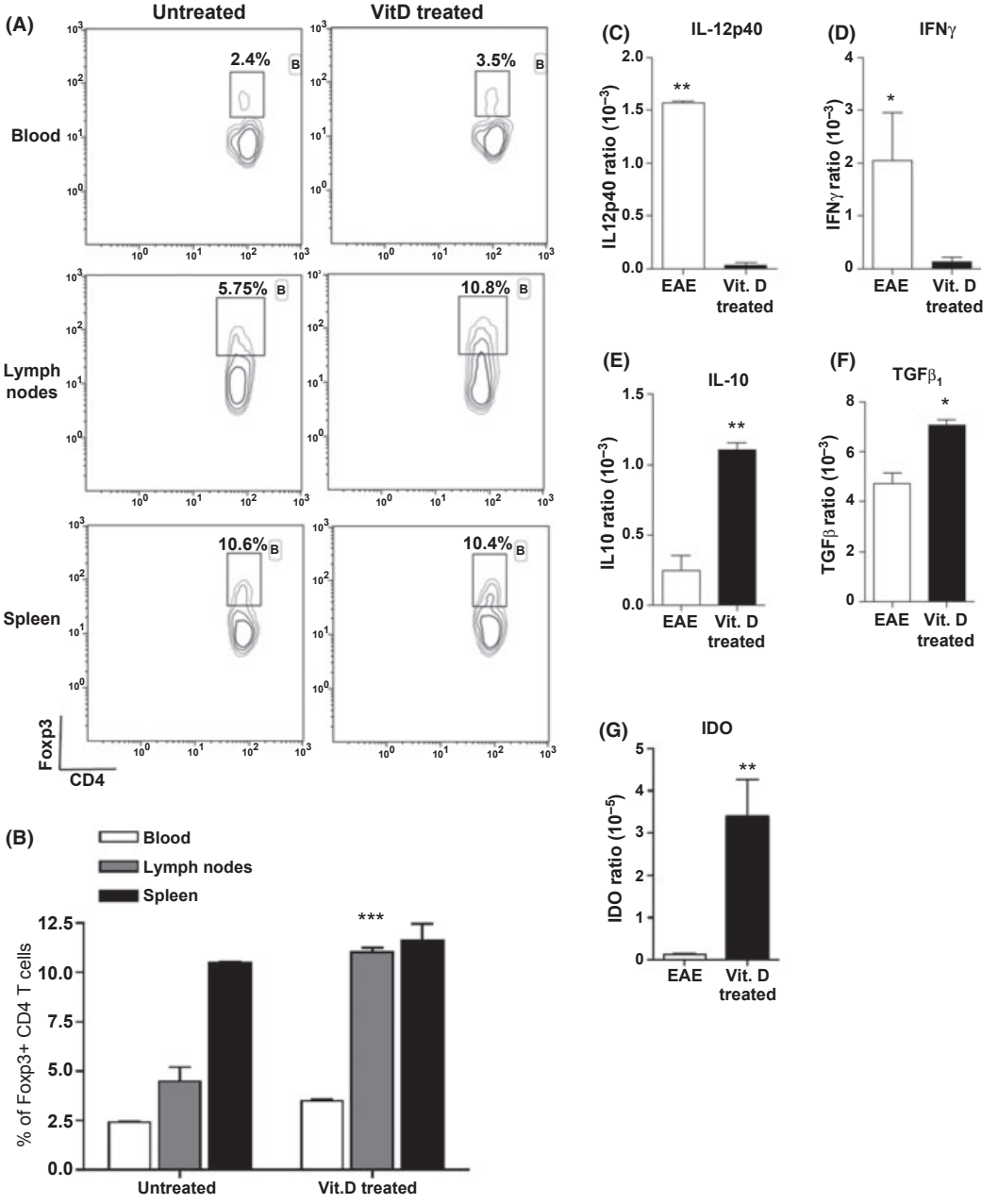

Figure 2 Vitamin D_3 treatment enhances the $CD4^+Foxp3^+$ T-cell population in the lymph nodes. *In vivo* treatment with vitamin D_3 increased the percentage of $CD4^+Foxp3^+$ T cells (10.8%) in the lymph nodes 12 days after immunization in relation to untreated animals (5.75%) (**A**). No significant difference in the percentage of $CD4^+Foxp3^+$ T cells in the blood or spleen was found between treated and untreated groups (**A** and **B**). Concomitant with the increase in $CD4^+Foxp3^+$, there is a significant decrease in the expression of IL-12p40 (**C**) and IFNγ (**D**) in the lymph nodes. The decrease in the expression of IL12/INFγ is accompanied by significant increases in IL-10 (**E**), TGFβ_1 (**F**), and IDO (**G**) expression. ** $P < 0.05$, **$P < 0.01$, ***$P < 0.001$*

decreased significantly ($P < 0.05$) in the sera from rats treated with vitamin D_3 (Figure 1B). The proliferative response of the lymph node cells upon stimulation with $gpMBP_{73\text{-}86}$ (10 μg/mL) was also significantly reduced in animals treated with vitamin D_3 (13,053 $\pm$ 1,328 cpm) compared with the untreated control group (32,321 $\pm$ 1,528 cpm) ($P < 0.001$) (Figure 1C). Yet, we found a slight decrease in CD80 expression in the lymph nodes of those animals (Figure S1 B). These results suggest an immunomodulatory effect of vitamin D_3 on neuroantigen-specific T- and B-cell responses.

In parallel, we found a significant increase in the production of both IL-10 ($P < 0.01$) and TGFβ_1 ($p < 0.05$) in the serum of rats treated with vitamin D_3 (Figure S1 C).

Analysis of Inflammatory Cell Infiltration of CNS

To confirm the protective effect of vitamin D_3 in EAE, histological analyses of central nervous tissue were performed. Both the number of inflammatory foci and the number of mononuclear cells infiltrating the CNS were evaluated. Figure 1D clearly demonstrates that fewer mononuclear cells infiltrated the CNS tissue in animals treated with vitamin D_3 than in untreated animals. However, when the number of inflammatory foci was quantified, no significant difference was found between the untreated and vitamin D_3-treated groups (Figure 1E). To confirm the histology data, the number of $CD3^+CD4^+$ cells in the spinal cord was quantified by flow cytometry using a known concentration of PE-beads. Figure 1F shows the number of $CD3^+CD4^+$ infiltrating cells that in the untreated group is almost twice that in the vitamin D_3-treated group. To investigate whether the lymphocytes that reached the CNS were functionally activated, markers of T-cell activation such as IL2R (CD25), TCR$\alpha\beta$, and OX40 were evaluated in the cells that infiltrated the CNS in animals treated with vitamin D_3 and in untreated animals. No differences in the expression of the activation molecules were observed in the two groups of animals (Figure S2 A). Additional experiments were conducted to investigate whether vitamin D_3 acts on cytokine production in the supernatant of homogenized spinal cords. A significant decrease in the production of IL-17A ($P < 0.05$), IFNγ ($P < 0.05$), and TNFα ($P < 0.0$ 1) was observed in the group of rats treated with vitamin D_3 (Figure S2 B).

Increase in Expression of $Foxp3^+$ Regulatory T cells in Lymph Nodes After Treatment with Vitamin D_3

The reduction in the proliferative response of autoreactive T lymphocytes and in the production of antibodies against MBP, accompanied by a simultaneous increase in IL-10 and TGFβ_1, after treatment with vitamin D_3 suggests the activation of regulatory T cells because these cells express mainly IL-10 and TGFβ_1 [33,34].

To investigate the participation of $Foxp3^+$ regulatory T cells in the immunomodulatory mechanism of vitamin D_3 in EAE, the expression of Foxp3 was evaluated in $CD4^+$ T cells from blood, spleen, and lymph nodes. In the lymph nodes, the presence of $Foxp3^+$ regulatory T cells was investigated 12 days after immunization. We observed a significant increase in the phase of exacerbation of the disease (12 d.a.i.) of $CD4^+Foxp3^+$ in the vitamin D_3-treated animals in the draining lymph nodes (10.8% of $CD4^+$ cells) versus the untreated controls (5,75% of $CD4^+$ cells). No significant difference in the number of $CD4^+Foxp3^+$cells in the blood or spleen was found for the two groups of rats studied (Figure 2A, B). All $CD4^+Foxp3^+$ cells were also tested for CD25, and almost all of the cells were CD25 positive (data not shown). These results strongly suggest an important role for regulatory T cells in the immunomodulatory mechanism of vitamin D_3 treatment in EAE, consistent with the *in vitro* observation of vitamin D_3 enhancement of regulatory T cells [20,22].

Vitamin D_3 Treatment and Cytokine Profile in the Lymph Nodes

Previous studies have demonstrated that $Foxp3^+$ regulatory T cells release large amounts of IL-10 and TGFβ_1 [34]. To determine whether treatment with vitamin D_3 stimulated the IL-10- and TGFβ_1-producing cells in the lymph nodes, the expression of these cytokines was investigated. The results demonstrated that treatment with vitamin D_3 induces significant IL-10 expression and a moderate increase in TGFβ_1 expression (Figure 2E, F). Moreover, we found a suppressive effect of vitamin D_3 treatment on the IL-12/IFNγ axis (Figure 2C, D), which was reported previously [14]. Interestingly, along with the increase in IL-10 and TGFβ_1, we found a significant increase in the IDO (Figure 2G). IDO is highly expressed in the DCs and plays an important role in the enhancement of regulatory T cells [35,36].

The Effect of Vitamin D_3 in the Generation of Bone Marrow-derived DCs

Dendritic cells have many effects on T-cell activation and control. There is strong evidence of the action of vitamin D_3 in activating the tolerogenic properties of DCs. To investigate this issue, bone marrow cells were cultured with GM-CSF or GM-CSF plus vitamin D_3 (Figure 3A). The results demonstrated that at least 70% of cells cultured in the presence of GM-CSF or GM-CSF and vitamin D_3 expressed DC markers after 12 days in culture. However, the cells that were cultivated in the presence of vitamin D_3 contained more $CD11b^+$ cells and fewer $CD11b^+CD11c^+$ and $CD11c^+$ cells than the control group (Figure 3B). *In vitro* treatment with vitamin D_3 was also able to induce a DC population (vdDCs) that expresses significantly less MHC-II and CD80 molecules with no changes in MHC class I molecules (Figure 3C, D). The vdDCs also showed a significant increase in the expression of TNFα and IL-10 (Figure 3E, F) in relation to the normal controls. No difference in the expression of IL-12 or TGFβ_1 was observed (Figure 3G, H). vdDCS were able to enhance $CD4^+Foxp3^+$ from $CD4^+$ from spleen cells, in larger percentage in comparison with control DCs (data not shown). These results suggest that vitamin D_3 enhances a tolerogenic/immature profile in DCs. To investigate the participation of IDO in the mechanism of vdDCs profile, we evaluated the expression of the transcription factor STAT3, which binds directly in the IDO promoter region [37]. Indeed, our results show a significant increase in STAT3 and IDO expression in vdDCs in comparison with control DCs (Figure 4A–C). These

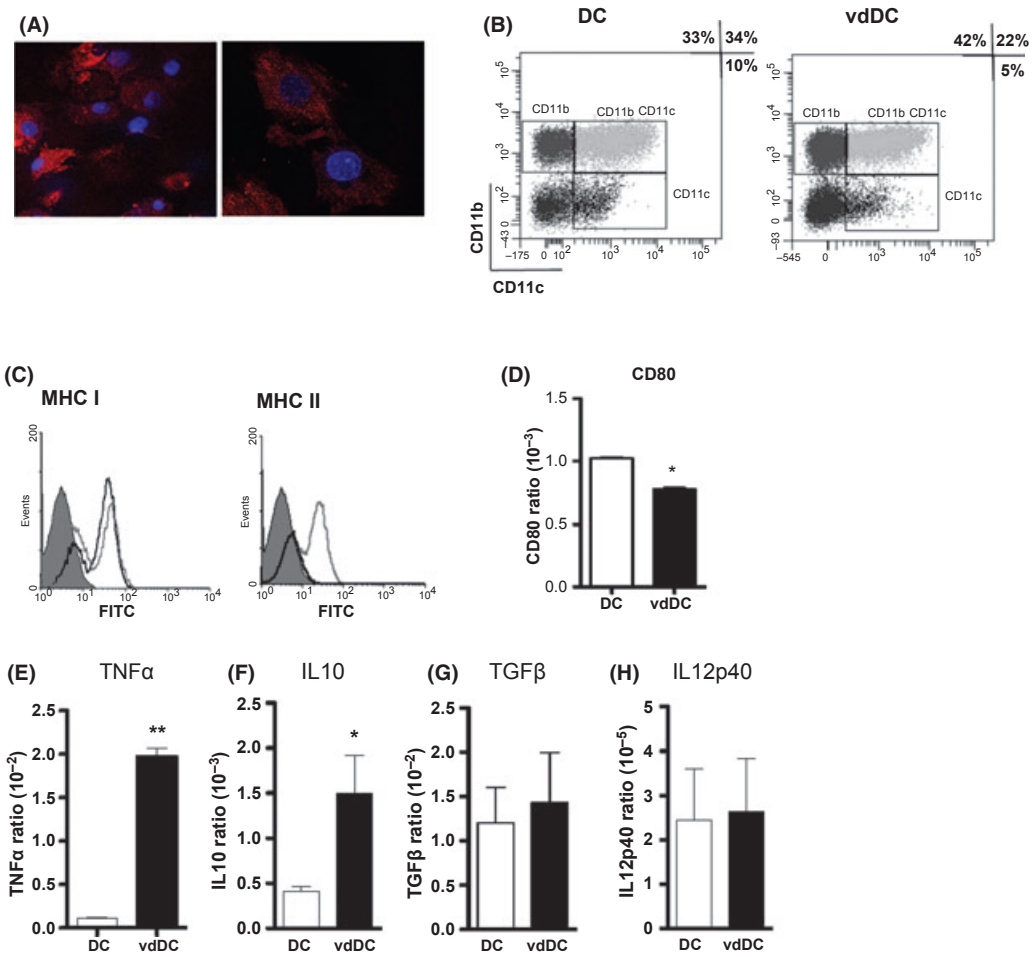

Figure 3 Bone marrow-derived DCs cultivated in the presence of vitamin D_3 exhibit a tolerogenic state. Bone marrow-derived DCs morphology. The nucleus is labeled with DAPI (blue), and the cytoplasm is labeled with PHK-26 (red), Confocal imaged using a 63x (left image) or 100x (right image) objective lens (**A**). Bone marrow cells cultured in the presence of GM-CSF and vitamin D_3 (vdDC) or GM-CSF (DC) alone present markers (CD11b and CD11c) for dendritic cells. However, the cells that were cultured in the presence of vitamin D_3 include a greater proportion of $CD11b^+$ or $CD11b^+CD11c^+$ cells and a lower proportion of $CD11c^+$ cells relative to control DCs (**B**). Flow cytometer analysis shows a low expression of MHC-II in vdDCs in relation to DCs (**C**). Concomitant with the decrease in MHC-II expression, real-time PCR shows a decrease in CD80 molecule in vdDCs in relation to control DCs (**D**). Real-time PCR analysis shows that TNFα and IL-10 expression are increased in the vdDCs (**E** and **F**), but there is no change in the expression of TGFβ_1 (**E**) or IL-12p40 (**F**). ** $P < 0.05$, **$P < 0.01$, ***$P < 0.001$*

results suggest that IDO has an important role in the tolerogenic profile of vdDCs. In fact, when cells were treated with an IDO inhibitor (L-methyl-tryptophan), the expression of IL-10 and TNFα decreased to levels comparable to those of control DCs (Figure 4D, E). Moreover, *in vivo* inhibition of IDO using L-methyl-tryptophan abolishes the beneficial effects of vitamin D_3 on the course of EAE (Figure 4F).

In Vivo DC and vdDC Transfer

Normal and vitamin D_3-induced DCs were adoptively transferred to Lewis rats 1 day prior to immunization with the encephalitogenic peptide. The transfer was performed by injection into the foot pad, in the same location as the actual immunization. This was considered appropriate because previous studies have observed that DCs can migrate into the popliteal lymph nodes when injected into the foot pad [38]. Our results provide evidence that the adoptive transfer of tolerogenic DCs significantly reduces the severity of EAE. These results are similar to those observed upon *in vivo* treatment with vitamin D_3 (Figure 5A).

Because the treatment with vitamin D_3 induced an increase in Foxp3 cells in the lymph nodes (Figure 2A, B), we investigated whether the vdDCs are involved in the process of regulatory T-cell activation. Therefore, the expression of Foxp3 in $CD4^+$ cells after the transfer of DCs or vdDCs was investigated. The adoptive transfer of vdDCs induced an increase in the percentage of $CD4^+Foxp3^+$ T cells in the lymph nodes (11.8%) at 12 days after immunization; this value was 5.2 and 6.2% for control and DC-transferred rats, respectively (Figure 5B). No change in the percentage of regulatory T cells was observed in either spleen or blood cells (data not

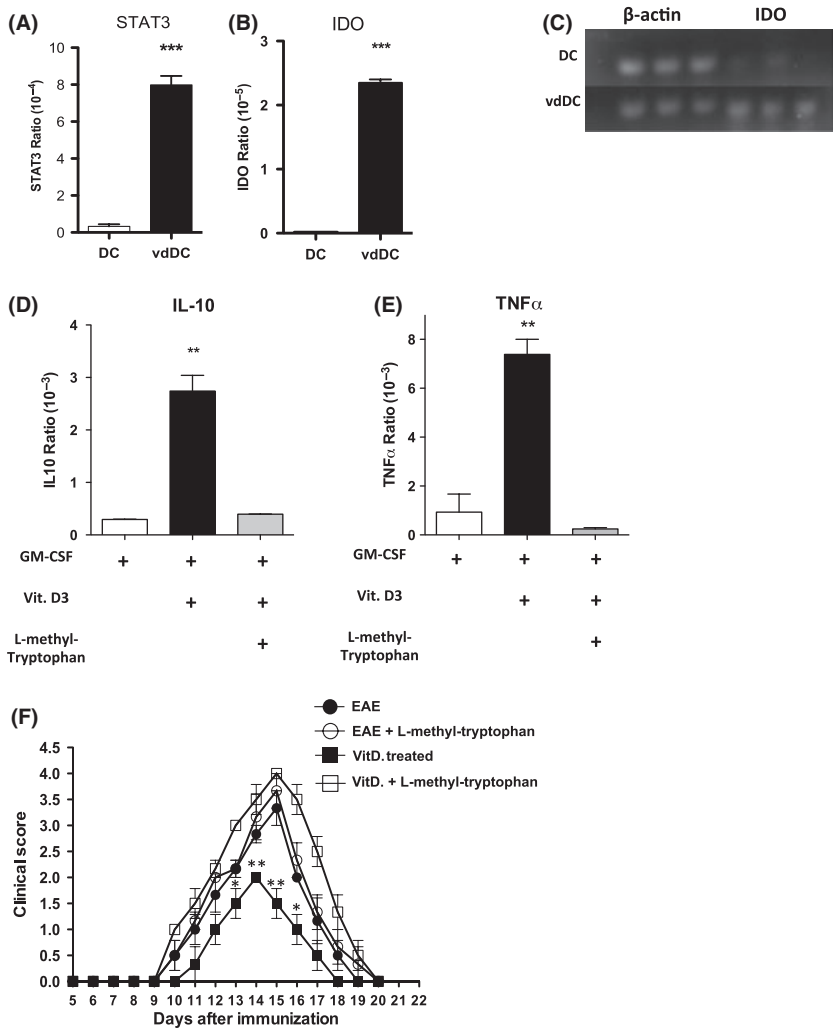

Figure 4 IDO is essential to the tolerogenic state of vdDCs. Real-time PCR analysis shows that vdDCs exhibit strong STAT3 (**A**) and IDO (**B**) expression. The expression of IDO in control DCs was undetectable in three samples; therefore, we ran a polyacrylamide gel to validate the controls (**C**). To verify the role of IDO in the tolerogenic state of vdDCs, we used a competitive inhibitor of the enzyme (L-methyl-tryptophan). The inhibition of IDO using L-methyl-tryptophan reverts the expression of IL-10 and TNFα by vdDCs (**D** and **E**, respectively). *In vivo* use of L-methyl-tryptophan (400 mg/Kg/day; orally) abrogates the beneficial effect of vitamin D_3 treatment (F). *$*P < 0.05$, $**P < 0.01$, $***P < 0.001$*

shown). The increase in percentage of $CD4^+Foxp3^+$ T cells observed after adoptive transfer of vdDCs is comparable to those observed after treatment with vitamin D_3 (Figure 2A).

These results clearly demonstrate that both vitamin D_3 treatment and vdDCs transfer led to a significant increase in the $Foxp3^+$ regulatory T-cell population in the lymph nodes. Moreover, there was a significant increase in the expression of IL-10 in the lymph node cells that adoptively received the vdDCs compared with those in the control group that received control DCs. No significant differences in the expression of TGFβ_1 were observed (Figure S3).

Discussion

During the last two decades, the immunomodulatory roles of vitamin D_3 and its active form (1, 25-dihydroxyvitamin D_3) in autoimmune disorders have become clear. However, the mechanisms that underlie these beneficial effects have not been fully elucidated. Here, we presented evidence that vitamin D_3 treatment enhances the development of $CD4^+Foxp3^+$ regulatory T cells and ameliorates the clinical course of EAE. Moreover, our results show that DCs may be the preferential targets of the immunomodulatory effect of vitamin D_3. Here, we chose to work with the vitamin D_3, instead of its active form $1,25(OH)_2D_3$; however, the reduction in EAE and the increase in the expression of Foxp3, IDO, and IL-10 and the generation of tolerogenic DCs were confirmed in the treatment with $1,25(OH)_2D_3$.

The presence of vitamin D_3 promoted a tolerogenic/immature state in bone marrow-derived DCs, as demonstrated by the down-regulation of the expression of CD80 and MHC-II molecules and concomitant significant increase in IL-10 and TNFα expression. These results may be explained, at least in part, by the high expression of STAT3 and IDO in those cells [37]. Recent reports have shown that vitamin D_3 treatment enhances IDO expression [21]. IDO is a tryptophan-degrading enzyme. The downstream metabolites of tryptophan suppress T-cell response, resulting in immunosuppression and tolerance. Although IDO can be expressed in numerous cell types, DCs appear to be the main cells that express this enzyme in the immune system. IDO^+ DCs, with a tolerogenic profile, exert both direct and indirect inhibition of immune response [39]. Here, the adoptive transfer of IDO^+ DCs, generated in the presence of vitamin D_3, ameliorates the clinical

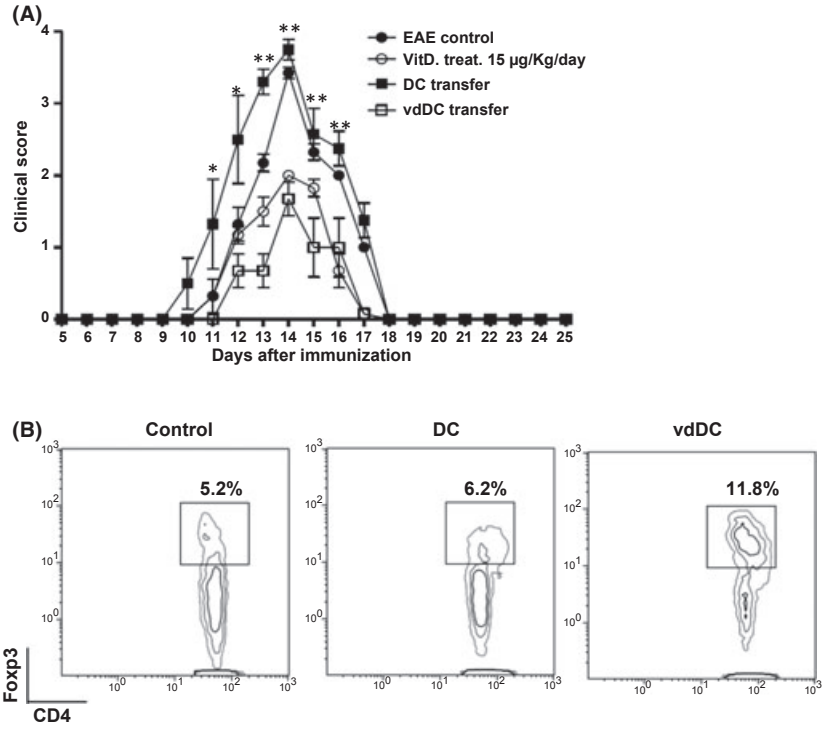

Figure 5 Enhancement of $CD4^+Foxp3^+$ after vdDC transfer. The transfer of vdDCs (white square) ameliorates the severity of encephalomyelitis (EAE), comparable to vitamin D_3 treatment (white circle), in relation to EAE control (black circle) or control DCs transfer (black square) (**A**). The transfer of vdDCs enhanced the population of $CD4^+Foxp3^+$ cells in the lymph nodes in relation to control DCs or control EAE, 12 days after immunization (**B**), comparable with our results for vitamin D_3 treatment. ** P < 0.05, **P < 0.01, ***P < 0.001*

course of EAE. These results are comparable to those observed upon *in vivo* treatment with vitamin D_3, suggesting the probable effect of vitamin D_3 on lymph node DCs. As a consequence of vitamin D_3 administration, the lymph nodes become an IL-10- and TGFβ_1-rich microenvironment, which favors the conversion of naïve T cells to regulatory T cells and the maintenance of the tolerogenic status of DCs [40]. Antiinflammatory cytokines generated by the treatment inhibit both the production of proinflammatory cytokines by autoreactive T cells and neuroantigen presentation. Moreover, the absence of the essential amino acid tryptophan may suppress the lymphoproliferative response, and kynurenines produced by tryptophan metabolism may present a direct toxicity and induce apoptosis in autoreactive T cells [41]. These mechanisms contribute actively to the suppression of the inflammatory response in the periphery, reducing the inflammation and CNS demyelination observed in the EAE model.

Although the beneficial effects of vitamin D_3 or its active form are clear in many autoimmune experimental models, the benefits to patients with MS remain to be elucidated; no trials have evaluated long-term treatment in a large population. Moreover, many studies conducted in different populations worldwide have shown an important polymorphism in the vitamin D receptor of patients with MS 42, which may explain the variable response presented by patients with MS.

Taken together, the evidence presented here shows that the *in vivo* administration of vitamin D_3 significantly reduced the severity of EAE. Our data suggest that DCs are the main targets of vitamin D_3. The IDO produced by tolerogenic DCs enhances Tregs in the lymph node microenvironment, which results in the inhibition of encephalitogenic T-cell development and consequently less severe EAE.

Acknowledgments

The authors would like to acknowledge the assistance of Linda Gentry El-Dash in linguistic revision of the manuscript. This work was supported by grants from FAPESP (#2011/18728-5) and FAEPex UNICAMP. ASF was supported by CNPq/DAAD grant #290089/2004-2 and FAPESP grant #2012/01408-0; GSS, PBR, COFO, and AGA were supported by PIBIC scholarships; and FP and MPAS were supported by FAPESP grants (#2011/15175-5, #2011/15639-1).

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. (A) Oral (2.5 and 5μg/Kg/day) and intraperitoneal (10 and 15 μg/Kg/day) vitamin D_3 treatment of EAE, no significant difference was found between the treatments and EAE control group. (B) There is a slight but significant decrease in the expression of CD80 in the lymph nodes of vitamin D_3-treated animals compares to untreated animals. (**C**) ELISA was used to measure the cytokine (IFNγ, TNFα, IL-10 and TGF_1) levels in the serum of vitamin D_3-treated (black bars) and untreated animals (white bars). There is a significant increase of IL-10 and TGFβ in the vitamin D_3-treated in relation to untreated animals * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure S2. (A) The expression of activation markers (IL-2R, OX40 and TCR) in mononuclear cells extracted from CNS after a Percoll gradient. (**B**) ELISA was used to measure the cytokine (IL-17A, IFNγ, TNFα, IL-10 and TGF_1) levels in the CNS supernatants of vitamin D_3-treated (black bars) and untreated animals (white bars). There is a significant decrease of IL-17A and IFNγ in the vitamin D_3-treated in relation to untreated animals * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure S3. IL-10 and TGFβ_1 expression, measured by real time PCR, in the lymph nodes 12 days after EAE immunization in untreated (white bars), vitamin D_3-treated (black bars), DC-transferred (light gray bars) and vdDC-transferred animals (dark gray bars). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.