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1,25-dihydroxyvitamin D_3 suppressed experimental autoimmune encephalomyelitis through both immunomodulation and oligodendrocyte maturation

Hasti Atashi Shirazi[*],
Javad Rasouli[*],
Bogoljub Ciric,
Danmeng Wei,
Abdolmohamad Rostami,
Guang–Xian Zhang[#]
Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107

Abstract

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has recently been found to have the anti-inflammatory potential to suppress experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis; however, its direct effect on neural cells is not clear. In the current study we show that $1,25(OH)_2D_3$ treatment effectively suppressed clinical signs of ongoing EAE and reduced inflammation and demyelination scores in the central nervous system (CNS). The treatment significantly decreased production/expression of pro-inflammatory cytokines IFN-γ, GM-CSF and IL-17A, while it increased anti-inflammatory cytokines IL-4 and IL-10. Further, $1,25(OH)_2D_3$ treatment effectively elevated the numbers of neural stem cells, oligodendrocyte precursor cells, as well as oligodendrocytes in disease lesions in the CNS. These results, together with its in vitro effect of inducing oligodendrocyte differentiation as shown in our previous findings, demonstrate that $1,25(OH)_2D_3$ suppressed EAE not only by its immunomodulatory capacity, but also by its effect on oligodendrocyte differentiation and maturation, and thus has potential for remyelination and neural repair.

Keywords

1, 25-dihydroxyvitamin D_3; Oligodendrocytes; Multiple Sclerosis; EAE

[#]Correspondence. guang-xian.zhang@jefferson.edu.
[*]Equal contribution

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) (Steinman 2001), whose progression is associated with extensive inflammatory infiltrates and relatively early loss of oligodendrocytes (OLGs), the myelinating cells, in the CNS (Lucchinetti, Bruck et al. 2001, Cudrici, Niculescu et al. 2006). While the etiology of MS is not well understood, the prevalence of this disease is increasing due to a complex interplay between environmental and genetic risk factors in susceptible individuals (Grytten, Glad et al. 2006, Compston and Coles 2008). Experimental autoimmune encephalomyelitis (EAE), an animal model of MS, makes it possible to explore multiple facets of the immune and neural mechanisms of disease, and to test interactions among a variety of mechanisms that lead to key pathological features of MS such as inflammation, demyelination, axonal loss and gliosis (Constantinescu, Farooqi et al. 2011, Lucchinetti, Bruck et al. 2001).

The activated form of vitamin D, 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) is an environmental factor that can modify multiple functions in the body, and a low level of this vitamin in the diet or low sunlight exposure increases the risk of MS (Munger, Levin et al. 2006, Wergeland, Torkildsen et al. 2011). Clinical effects of $1,25(OH)_2D_3$ are mediated via the vitamin D receptor (VDR), which is widely distributed both in immune cells and CNS resident cells (Mayne, Spanier et al. 2011, Cekic, Sayeed et al. 2009). $1,25(OH)_2D_3$ predominantly mediates immunomodulatory responses in vitro and in vivo (Dehghani, Meamar et al. 2013, Smolders, Thewissen et al. 2009), and effectively suppresses EAE (Cantorna, Humpal-Winter et al. 2000, Smolders, Thewissen et al. 2009, Becklund, Hansen et al. 2009). On the other hand, VDR expression in OLGs may play an important role in maintaining a balance between OLG differentiation and axonal adhesion during brain development, while VDR depletion leads to a slower rate of OLG differentiation with an increased proportion of apoptosis in these cells (Chaudhuri 2005). Our previous study showed that $1,25(OH)_2D_3$ in vitro significantly enhanced proliferation of neural stem cells, and promoted their differentiation into neurons and OLGs, but not astrocytes, likely through inducing production of neurotrophic factors NT-3, BDNF, GDNF and CNTF (Shirazi, Rasouli et al. 2015). However, the effect of this vitamin on OLG differentiation in EAE is not known.

In the present study, we investigated the therapeutic potential of $1,25(OH)_2D_3$ in EAE by studying its effect on cytokine regulation and neuron/OLG differentiation whereby demyelination was reduced in EAE/MS.

Materials and Methods

EAE induction and treatment

Female C57BL/6 mice, 8–12 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, Maine) and used for EAE induction. EAE was induced by subcutaneous injection of 200 μg MOG_{35-55} (Gen Script, Piscataway, NJ) in complete Freund's adjuvant (CFA) containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Mice received 200 ng pertussis toxin (Sigma Aldrich, St. Louis, MO) intraperitoneally (i.p.) on days 0 and 2 post immunization (p.i.). Daily clinical observation and grading were recorded by two

researchers in a blind manner according to a 0 to 5 scale as follows : 0, clinically normal; 1, limp tail or waddling gait with tail tonicity; 2, waddling gait with a limp tail (ataxia); 2.5, ataxia with partial limb paralysis; 3, full paralysis of one limb; 3.5, full paralysis of one limb with partial paralysis of a second limb; 4, full paralysis of two limbs; 4.5, moribund; and 5, death. All work was performed in accordance with the guidelines for animal use and care at Thomas Jefferson University.

EAE mice received i.p. injection of 0.1 μg of 1,25 $(OH)_2D_3$ (Sigma Aldrich) along with 0.1% ethanol in a volume of 0.2 ml of normal saline, every second day from day 15 to 30 p.i., as previously described (Chaudhuri 2005, Dehghani, Meamar et al. 2013). Mice that received the same volume of normal saline with 0.1% ethanol base served as control.

Histological analysis

On day 30 p.i., mice were extensively perfused with PBS, and lumbar spinal cords were harvested and fixed with 4% PFA (Mediatech, Inc., Manassas, VA). The five-micrometer sections of tissues were stained with hematoxylin and eosin (H&E) to determine inflammatory infiltrate cells and with luxol fast blue (LFB) to examine demyelination. Slides were assessed for inflammation and demyelination by two researchers in a blinded manner, as follows. For inflammation: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. For demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination.

Cell culture and flow cytometry

Splenocytes were harvested at day 30 day p.i and cultured with MOG_{35-55} (25 μg/ml) at a density of 2×10^6/ml in cell media (IMDM, 5% FBS, Pen-Strep, L-Glutamin) for 3 days. For intracellular cytokine secretion, cells were activated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), ionomycin (500 ng/ml) and Golgi Plug (1 μg/ml) (BD Biosciences, San Jose, CA) for 4 hours. Cells were washed and stained with CD4 Pacific Blue (BD Biosciences) and CD8 Percp-Cy5.5 (BD Biosciences), and fixed/permeabilized with Caltag Fix/Perm reagents (Invitrogen). Afterwards, cells were stained for intracellular cytokines with the following antibodies: IL-4-FITC, IL-5-allophycocyanin, IL-10-PE, IL-17A-Alexa Fluor 488, GM-CSF-PE and IFN-γ-allophycocyanin; appropriate isotype antibodies were used as control (all from BD Biosciences). Data were evaluated by FACS Aria II (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (Tree Star).

ELISA and cytokine quantification

Supernatants were harvested 3 days after culture; concentrations of IFN-γ, IL-17A, GM-CSF, IL-4, and IL-10 were quantified by ELISA (R&D system, Minneapolis, MN) according to the manufacturer's instructions.

Real-time quantitative PCR

Total RNA was extracted from spinal cord tissues (RNeasy Kit, QIAGEN Valencia, CA). RNA concentration and purity were determined (NanoDrop Technologies, Wilmington, DE); cDNA was then synthesized from 1 μg of total RNA according to the manufacturer's

instructions (QuantiTect Reverse Transcription Kit, QIAGEN, Valencia, CA). RT-PCR reactions (QuantiFast SYBR Green PCR Kit, QIAGEN, Valencia, CA) were initiated with denaturation at 95°C for 10s, annealing at 60°C for 30s and polymerization at 72°C for 30s with 40 cycles. The experiment was carried out in triplicate for cytokine gene expression, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene primer serving as an endogenous control and internal standards. Primer sequences (Integrated DNA Technologies, Coralville, Iowa) are shown in Table 1. Real-time qRT-PCR was performed with ABI Prism 7000 sequence detection system (Applied Bio-Systems, Foster City, CA).

Immunohistochemistry

Lumbar spinal cords of $1,25(OH)_2D_3$ treated and untreated mice were harvested (n=5 each group) at day 30 p.i. and fixed with 4% paraformaldehyde (Mediatech, Inc., Manassas, VA). Paraffin embedded tissues were cut into 5-μm thick sections. Immunohistochemistry staining was performed on three serial sections of spinal cords per mouse. Sections were deparaffinized/dehydrated with xylene and ethanol and washed with PBS. Samples were blocked with 8% horse serum and 3% BSA in PBS for 30 minutes at room temperature and incubated with the following primary antibodies at 4°C overnight: anti-nestin (1:500), anti-GalC (1:50), anti-NG2 (1:300), anti-MBP or myelin basic protein (1:100), anti-CD4 (1:500), anti-CD11b (1:400) and anti-VDR (1:50). Sections were washed and incubated with secondary antibody (1:500, Alexa Fluor 488 X IgG H&L; all of the above from Abcam, Cambridge, MA, USA) for 1 hour at room temperature. Serial sections of each sample were used as negative controls by using only secondary antibodies. Sections were washed and mounted with ProLong Diamond Antifade mounting media (Life Technologies, Grand Island, NY) and visualized by fluorescence microscope (Nikon Eclipse E600, Optical Apparatus Co. Dewitt, Iowa), (Helming, Bose et al. 2005, Yang, Yan et al. 2010). Mean pixel intensity of biomarker expressions and the number of CD4 and CD11b cells in lesion areas were quantified according to “counting of single color image” method by Image J software (Adzemovic, Zeitelhofer et al. 2013; Yang, Yan et al. 2014).

Statistical analysis

Clinical scores were analyzed by calculating the area under the curve for each mouse over the clinical period of the experiment. Differences between multiple groups were evaluated by the Kruskal-Wallis one-way analysis of variance. Experiments with two groups for statistical significance were tested by using unpaired, two-tailed, Student’s *t*-tests and Two-way ANOVA. $P< 0.05$ was considered to be statistically significant. Experimental data are presented as the mean ± SEM of three independent experiments. Graphs of the original data were produced using GraphPad Prism software, version 6.00 for Windows GraphPad, USA.

Results

1, 25 $(OH)_2D_3$ suppresses inflammation and demyelination in EAE.

To evaluate the effect of $1,25(OH)_2D_3$ on remyelination in vivo, EAE was induced and mice received $1,25(OH)_2D_3$ at day 15 p.i and for additional 15 days. Vehicle-treated mice developed chronic EAE and $1,25(OH)_2D_3$ significantly reduced clinical scores and halted

disease progression (Fig. 1A). Next, we sacrificed the mice at day 30 and the lumbar part of spinal cords was analyzed for EAE-associated CNS pathology. Our data demonstrate that $1,25(OH)_2D_3$ significantly reduces inflammation (Fig. 1B) and demyelination (Fig. 1C) when compared with controls.

Our histopathology results demonstrate that $1,25(OH)_2D_3$-treated mice had less inflammation in the white matter of the spinal cord. To analyze infiltrating cells, we stained serial sections of the lumbar part of spinal cord for CD4 and CD11b. Our immunohistochemistry results show that $1,25(OH)_2D_3$ significantly decreased the numbers of both infiltrating $CD4^+$ (Fig. 2A) and $CD11b^+$ cells (Fig. 2B) when compared to the vehicle group.

$1,25(OH)_2D_3$ enhances expression of immunoregulatory cytokines and decreases pro-inflammatory cytokines in EAE.

Next, we evaluated the effect of $1,25(OH)_2D_3$ on cytokine profile in the secondary lymphoid organs and the CNS of EAE mice. $1,25(OH)_2D_3$ significantly increased the percentages of myelin specific IL-4- and IL-10-producung $CD4^+$ T cells in the spleen of EAE mice. $1,25(OH)_2D_3$ treted mice had a lower proportion of IFN-γ^+, IL-$17A^+$, and GM-CSF^+ $CD4^+$ T cells compared with the vehicle group (Fig. 3A, B). We also measured cytokine production by myelin-specific splenocytes. Similar to flow cytometry results, $1,25(OH)_2D_3$ dramatically reduced production of pro-inflammatory cytokines such as IFN-γ, GM-CSF and IL-17A, while it induced production of IL-4 and IL-10 (Fig. 4A).

To determine the effect of $1,25(OH)_2D_3$ on cytokine levels in the CNS of EAE mice, total RNA was extracted from spinal cords and mRNA expression was quantified by real-time PCR. Consistent with cytokine production in splenocytes, $1,25(OH)_2D_3$ significantly enhanced the expression of IL-4 and IL-10, while it inhibited the expression of IFN-γ, GM-CSF and IL-17A (Fig. 4B). Together, these results indicate that $1,25(OH)_2D_3$ has an immunomodulatory effect in EAE.

$1,25(OH)_2D_3$ treatment enhances differentiation/survival of oligodendrocyte progenitor cell/oligodendrocyte (OPC/OLG) lineage

Our previous study showed that $1,25(OH)_2D_3$ promoted neural stem cell (NSC) proliferation and OPC/OLG lineage differentiation in vitro (Shirazi, Rasouli et al. 2015). To study the effect of $1,25(OH)_2D_3$ on NSC proliferation/survival and OPC/OLG differentiation in vivo, we stained the lumbar part of the spinal cord of EAE mice for NG2 (OPCs marker; Fig. 5A), GalC (OLGs marker; Fig. 5B), nestin (NSCs marker; Fig. 5C), and MBP (myelin sheath marker; Fig. 5D) and analyzed by immunofluorescence microscopy. $1,25(OH)_2D_3$-treated mice had higher expression levels of NG2, GalC, Nestin, and MBP in their spinal cords when compared with vehicle-treated mice (Fig. 5E). These results indicate that $1,25(OH)_2D_3$, similar to its effects in vitro, effectively induces NSC proliferation/survival and their differentiation into OPC/OLG lineage in vivo.

Discussion

The present study provides evidence that $1,25(OH)_2D_3$ treatment significantly suppressed ongoing EAE, through both its immunomodulatory capacity and direct promoting effect for the differentiation of NSCs into OPC/OLG lineage.

In addition to its fundamental role in calcium homeostasis and bone metabolism, $1,25(OH)_2D_3$ plays a particular immunoregulatory role in the treatment of autoimmune diseases. $1,25(OH)_2D_3$ skews pro-inflammatory Th1/Th17 cell-mediated cytokines to an anti-inflammatory Th2 cell-mediated state (Matheu, Back et al. 2003, Tang, Zhou et al. 2009, Helming, Bose et al. 2005). The administration of $1,25(OH)_2D_3$ can initiate a biological process that reduces the number of pathogenic CD4 cells and weakens their activity (Cantorna, Snyder et al. 2015), while a high vitamin D3 content is associated with attenuated white matter microglia activation/macrophage infiltration during OLG death and demyelination (Wergeland, Torkildsen et al. 2011, Huang, Ho et al. 2015). Moreover, the active metabolite of $1,25(OH)_2D_3$ exerts immunomodulatory effects by inhibiting differentiation of dendritic cells and desensitizing them to maturation stimuli (Griffin, Lutz et al. 2000). This process effectively controlled T cell responses and induced rapid apoptosis of inflammatory cells (Pombo, Barettino et al. 1999, Disanto, Sandve et al. 2012). These data, together with our observations in ongoing EAE, indicate that $1,25(OH)_2D_3$ is a potential therapy to inhibit Th1/Th17 cells and antigen presenting cells, thus suppressing the progression of EAE/MS.

We and others have shown that $1,25(OH)_2D_3$ reduces demyelination in EAE, an effect that can be explained by two possible mechanisms: First, demyelination in the CNS lesions of MS/EAE takes place in a pro-inflammatory environment that is intrinsically hostile to the OPC/OLG lineage (Franklin and Ffrench-Constant 2008). $1,25(OH)_2D_3$ may reduce autoimmune response by converting a hostile environment into an anti-inflammatory milieu that induces remyelination. Indeed, Th1 and Th17 cells play a central role in the development of EAE (Pikor, Prat et al. 2015) and are toxic to NSCs and OPCs (Li, Li et al. 2013, Helming, Bose et al. 2005). A pronounced suppression of IFN-γ and IL-17A by $1,25(OH)_2D_3$ treatment would therefore protect these cells from the harmful environment created by pro-inflammatory cytokines in the disease foci. On the other hand, although IL-4 does not directly prevent demyelination (Dumitrascu, Mott et al. 2014), increased IL-4 production by $1,25(OH)_2D_3$ treatment would indirectly contribute to remyelination through inhibiting Th1 and Th17 cells (Annunziato, Romagnani et al. 2015). Furthermore, the IL-10-IL-10R pathway is essential for suppression of EAE by $1,25(OH)_2D_3$ (Spach, Nashold, et al. 2006). $1,25(OH)_2D_3$ induces IDO^+ tolerogenic DCs and enhances Treg, reducing the severity of EAE (Farias, Spagnol, et al., 2013). In addition, $1,25(OH)_2D_3$ induces IL-10 production by splenocytes *in vitro* (Waddell, Zhao, et al., 2015). Our results indicate that $1,25(OH)_2D_3$ treatment increases IL-10 expression in the CNS and its production by splenic CD4 T cells. In addition to its anti-inflammatory effect, IL-10 also protects OLGs from inflammation-induced damage (Molina-Holgado, Grencis et al. 2001), and the increased IL-10 level induced by $1,25(OH)_2D_3$ may directly interact with resident (endogenous) OLGs and neurons locally to promote survival of these cells (Molina-Holgado, Grencis et al. 2001). Thus, the anti-inflammatory effects of $1,25(OH)_2D_3$ treatment can result

in a significantly increased number of OLGs, which provides a foundation for enhanced remyelination (Lin, Bailey et al. 2007).

The second possible mechanism of action of $1,25(OH)_2D_3$ on demyelination can be through its direct effect on OPC/OLG lineage. As a therapeutic factor, $1,25(OH)_2D_3$ can infiltrate into the CNS through the blood-brain barrier (BBB), thereby exerting a direct protective effect on neurons and OLGs (Zahir, Klassen et al. 2005, DeLuca, Kimball et al. 2013). $1,25(OH)_2D_3$ could also induce remyelinating through rapid proliferation of OPCs in response to injuries within the CNS (Gacias and Casaccia 2013, Li and Leung 2015). It has also been shown that $1,25(OH)_2D_3$ treatment increased OPC proliferation and OLG differentiation in demyelinated areas leading to remyelination (Gacias and Casaccia 2013). Direct proof that $1,25(OH)_2D_3$ promotes remyelination comes from a cuprizone-induced demyelination model, in which $1,25(OH)_2D_3$ actually promoted the repair process, possibly through a stimulating effect on OLG maturation and astrocyte activation (Nystad, Wergeland et al. 2014). Our own in vitro study demonstrated that $1,25(OH)_2D_3$ has a direct effect on NSC proliferation, survival, and neuron/OLG differentiation, through upregulating neurotrophic factors such as NT-3, BDNF, GDNF and CNTF (Shirazi, Rasouli et al. 2015), providing a mechanism for the observations in the present study in vivo. These results are also consistent with an observation showing that $1,25(OH)_2D_3$ can modulate the transcription of target genes to increase certain locally derived factors, such as basic fibroblast growth factor (Gagelin, Pierre et al. 1995) and, consequently, ciliary neurotrophic factor (Zahir, Klassen et al. 2005). Together, these observations provide a basis for a direct protective and remyelinating effect of $1,25(OH)_2D_3$ treatment in EAE.

It has been suggested that immunomodulation should be considered as a combinatorial therapeutic approach along with a strategy to protect OLGs and promote remyelination (Helming, Bose et al. 2005). Certain repurposed drugs are showing promise for protecting OLGs and for remyelination, and pharmacological targets have shifted from cellular replacement to endogenous repair enhancement (Helming, Bose et al. 2005). $1,25(OH)_2D_3$, in addition to its immunomodulatory capacity, could therefore be a potential neuroregenerative agent through promoting NSC differentiation into OPC/OLG linage and/or the survival of these cells.

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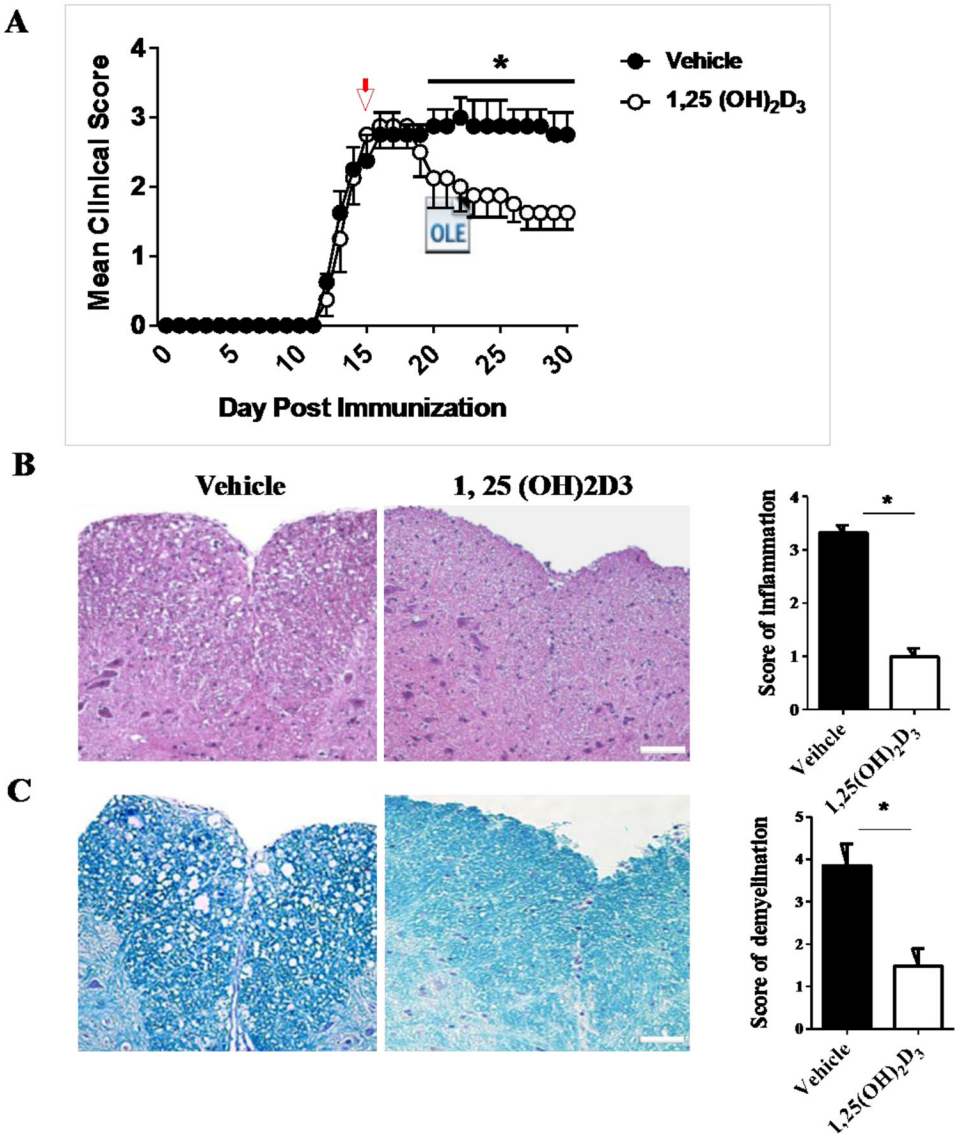

Figure 1. 1,25 $(OH)_2D_3$ ameliorates clinical signs of EAE and suppresses inflammation and demyelination in the CNS.

(A) Clinical EAE scores were evaluated daily according to a 0-5 severity scale in $1,25(OH)_2D_3$ treated and vehicle mice. Spinal cords were harvested at day 30 p.i. and stained with **(B)** H&E for inflammation and **(C)** Luxol fast blue (LFB) (for degree of demyelination. Scale bars= 20 μm. Data represent mean± SEM (n=5/group). *P<0.05. One representative of three independent experiments is shown.

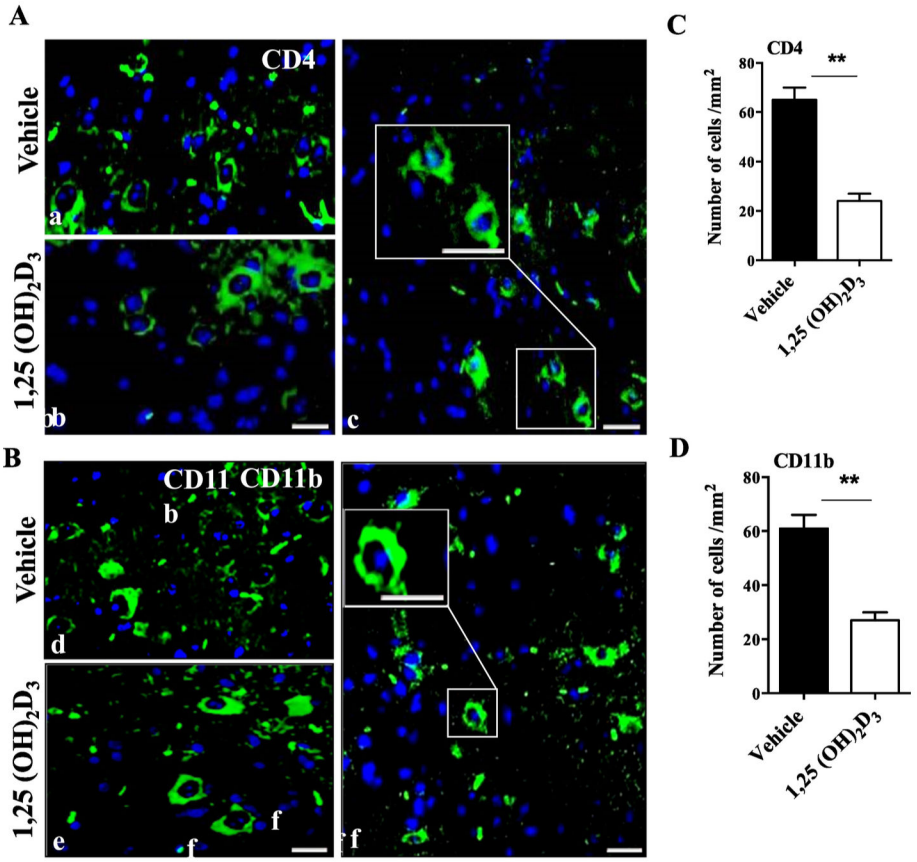

Figure 2. 1,25(OH)$_2$D$_3$ treatment reduces numbers of CD4$^+$ and CD11b$^+$ cells in the CNS.

Lumbar spinal cords of 1,25(OH)$_2$D$_3$ treated and vehicle EAE mice were harvested at day 30 p.i. for immunostaining. Representative images indicate **(A)** CD4$^+$ T cells and **(B)** CD11b$^+$ microglia/macrophages. Nuclei were stained with DAPI (blue). Quantitative analysis of numbers of **(C)** CD4$^+$ and **(D)** CD11b$^+$ cells per mm^2 in the spinal cord. Scale bars at c and f are 20 μm in small square and 40 μm in large square, respectively. Data represent mean ± SEM (n=5/group). **P< 0.01. One representative of three experiments is shown.

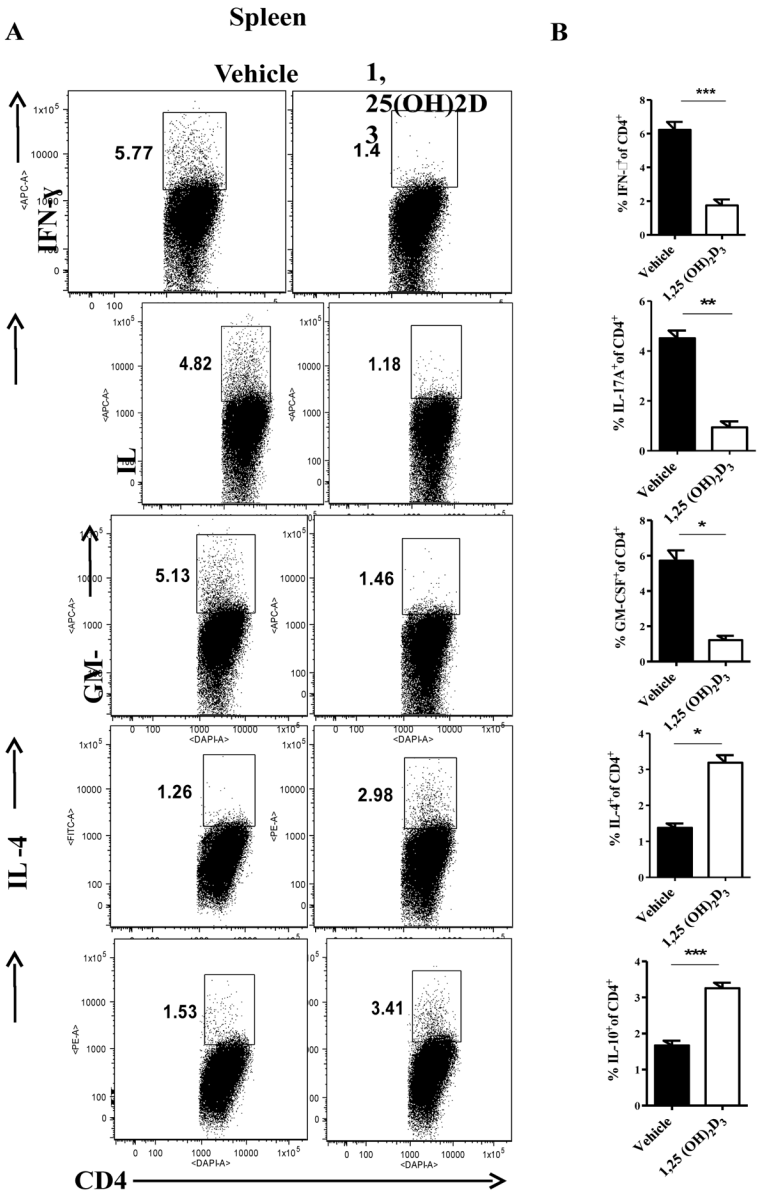

Figure 3. 1,25(OH)$_2$D$_3$ induces immunoregulatory cytokine and decreases pro-inflammatory cytokines expression in EAE.

Splenocytes of 25(OH)$_2$D$_3$ treated and vehicle EAE mice were stimulated with PMA, ionomycin and Golgi Plug for 4 hours, stained, and analyzed by flow cytometry. Percentages of IFN-γ^+, IL-17A$^+$, GM-CSF$^+$, IL-4$^+$ and IL-10$^+$ cells in gated CD4$^+$ T cells were determined **(A)**, and statistically analyzed **(B)**. Data represent mean±SEM (n=5/group), *P< 0.05, **P< 0.01, and ***P< 0.001. One representative of three experiments is shown.

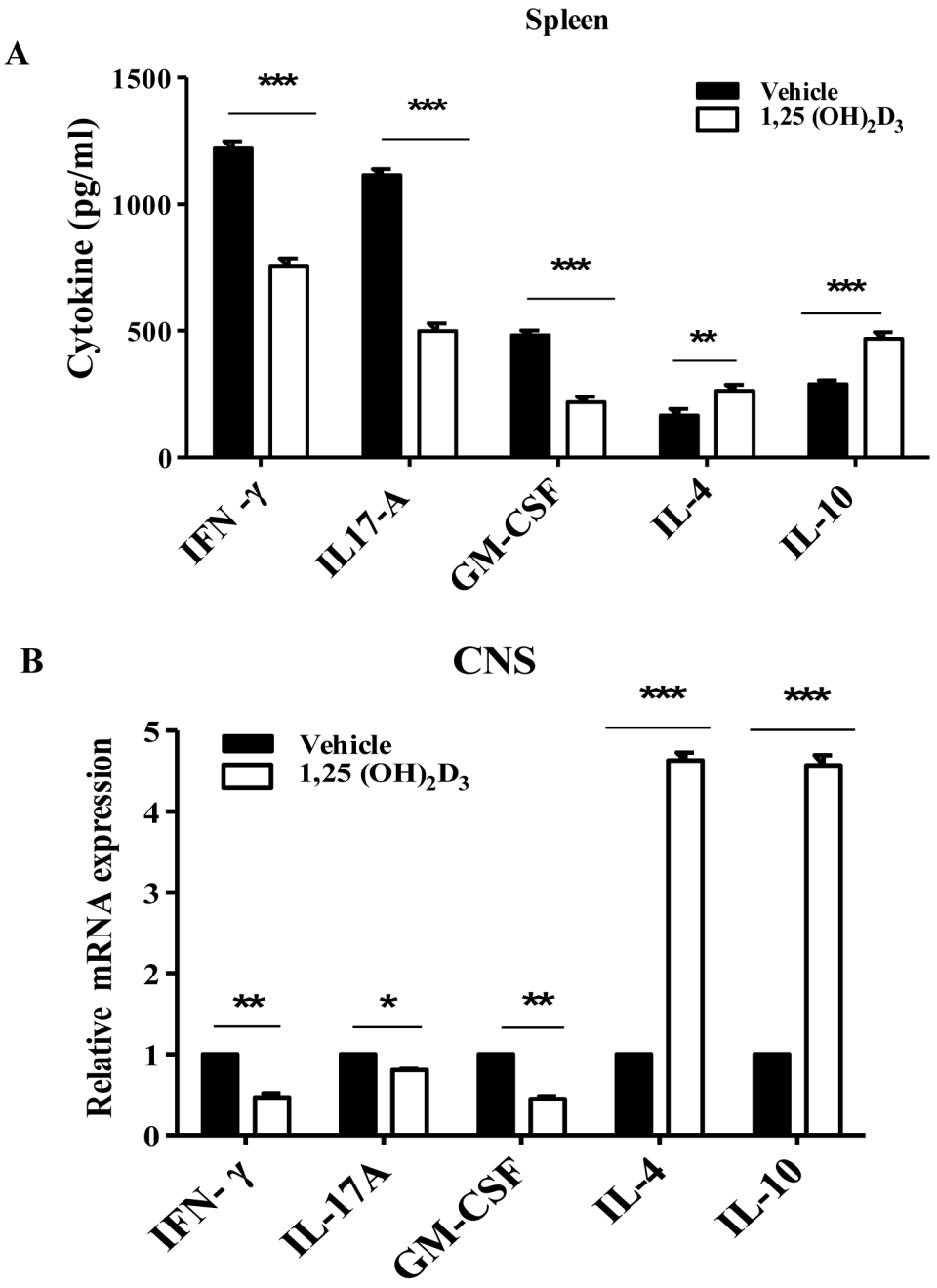

Figure 4. 1,25(OH)$_2$D$_3$ increases immunoregulatory cytokine production/expression.

(A) Splenocytes of 25(OH)$_2$D$_3$ treated and vehicle EAE mice were culture with MOG$_{35-55}$ for three days and supernatants were collected and analyzed for cytokine production by ELISA. **(B)** Spinal cord was collected and mRNA expression of IFN-γ, IL-17A, GM-CSF, IL-4 and IL-10 was quantified by RT-PCR. Data represent mean± SEM (n=5/group), *P<0.05, **P< 0.01, and ***p<0.001. One representative of three experiments is shown.

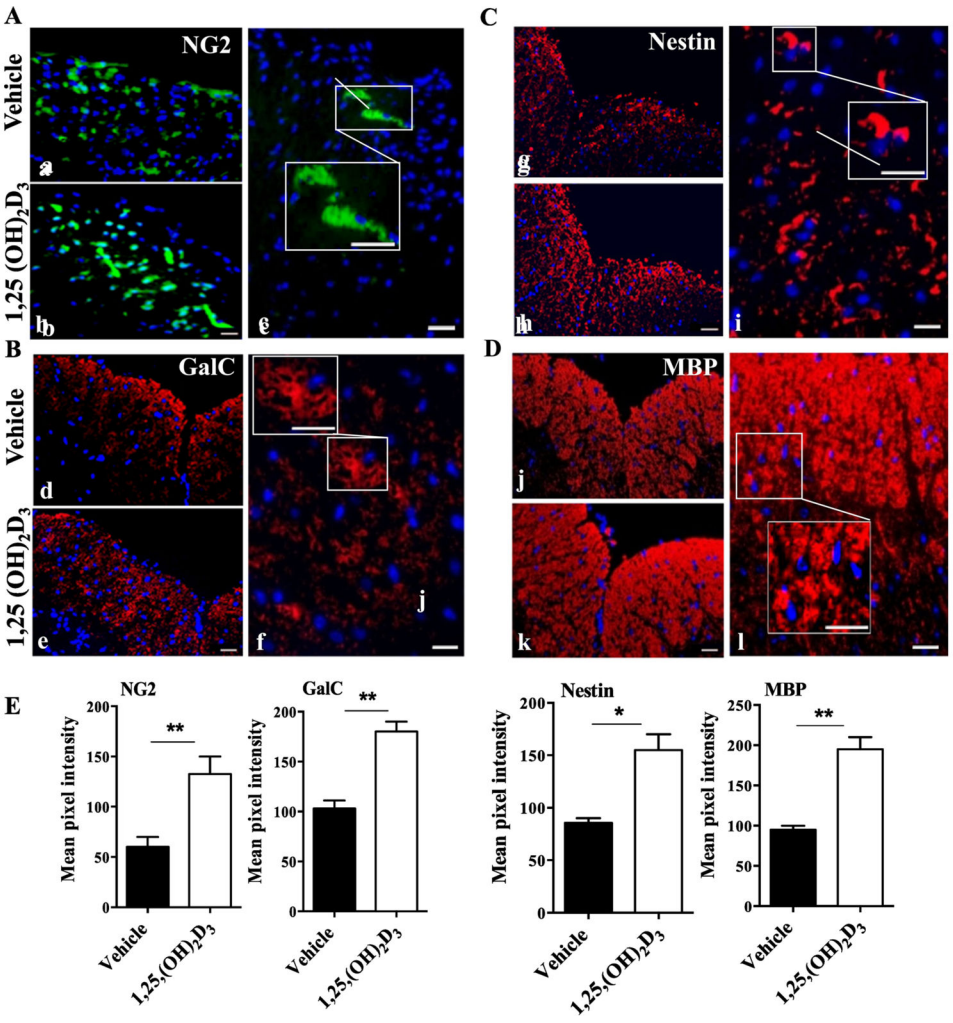

Figure 5. 1,25(OH)$_2$D$_3$ enhances remyelination.

Lumbar spinal cords of 25(OH)$_2$D$_3$ treated and vehicle EAE mice were harvested at day 30 p.i., and 3 sections from each mouse were stained and analyzed by confocal microscopy. Representative images indicate **(A)** OPCs (NG2$^+$; green), **(B)** OLGs (GalC$^+$; red), **(C)** neural stem cells (nestin$^+$; red), and **(D)** Myelin sheath (MBP$^+$, red). Nuclei were counterstained with DAPI (blue). **(E)** Image J was used to perform quantitative analysis for the pixel intensity of NG2, GalC, nestin and MBP at random areas of spinal cord lesions. Scale bars =10 μm, 20 μm, and 40 μm, respectively. Data represent mean± SEM (n=5 each group). *P<0.05 and **P< 0.01. One representative of three experiments is shown.

Table 1:

Sequences of primers used in real time RT-PCR

Gene Name	Primer sequence
IL-4	Forward : CGA GTT GAC CGT AAC AGA CAT Reverse : CGT CTT TAG CCT TTC CAA GAA G
IL-5	Forward : GGG CTT CCT GCT CCT ATC TA Reverse : CAG TCA TGG CAC AGT CTG AT
IL-10	Forward : CCC TGG GTG AGA AGC TGA AG Reverse : CAC TGC CTT GCT CTT ATT TTC ACA
IL-17A	Forward : ACT ACC TCA ACC GTT CCA CG Reverse : AGA ATT CAT GTG GTG GTC CAG
GM-CSF	Forward : GCT GCC CAA CCC TGT CAC C Reverse : GGT TGC CCC GTA GAC CCT GC
IFN-γ	Forward : TGT TAC TGC CAC GGC ACA GTC ATT Reverse : GTG GAC CAC TCG GAT GAG CTC ATT
GAPDH	Forward : ACC ACA GTC CAT GCC ATC AC Reverse : TCC ACC ACC CTG TTG CTG TA