Vitamin D Modulates the Expression of IL-27 and IL-33 in the Central Nervous System in Experimental Autoimmune Encephalomyelitis

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ABSTRACT

Background: It has been reported that vitamin D has broad anti-inflammatory and immunomodulatory effects. **Objective:** To evaluate the effects of vitamin D on the expression of IL-27 and IL-33 in a model of experimental autoimmune encephalomyelitis (EAE). **Methods:** EAE was induced in C57BL/6 mice by immunization with myelin oligodendroglial glycoprotein mixed with complete Freund's adjuvant. The mice were administered with PBS or olive oil, intraperitoneally, in the control groups and vitamin D (200 ng every two days) in the treatment group, from day +3 to +30. At day 31, the mice were scarified and their spinal cords and brains were harvested. The expression of the IL-27 and IL-33 mRNA in the spinal cord was measured using real time-PCR. **Results:** In PBS- or olive oil-treated EAE mice the expression of IL-27 P28 mRNA was significantly lower than that in the healthy control group ($p<0.002$). In both PBS- and olive oil-treated EAE groups, the expression of IL-27 EBI3 mRNA was also lower than that observed in the healthy group, but the differences were not significant. In vitamin D-treated EAE group, the expression of IL-27 P28 and IL-27 EBI3 were significantly higher compared with the olive oil-treated EAE groups ($p<0.002$ and $p<0.04$, respectively). The expression of IL-33 was significantly higher in PBS-or olive oil-treated EAE groups compared with healthy mice ($p<0.05$ and $p<0.02$, respectively). Vitamin D significantly decreased the expression of IL-33 compared with PBS- or olive oil-treated EAE mice ($p<0.04$, $p<0.02$, respectively). The PBS- or e oiloliv-treated EAE mice showed the clinical symptoms of EAE at days 9 and 10, respectively. The vitamin D-treated EAE group exhibited the symptoms at day 12 post immunization. The maximum mean clinical score and mean pathological scores were also significantly lower in vitamin D-treated EAE group, in comparison with PBS- or olive oil treated EAE mice ($p<0.001$). **Conclusion:** Vitamin D may modulate the expression of IL-27 and IL-33 in the spinal cord of EAE mice and also ameliorate the clinical symptoms of the disease.

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INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS), with a higher prevalence rate in females than males, more especially in those living in high altitudes with low sun light exposure (1). The experimental autoimmune encephalomyelitis (EAE), an animal model of MS, is inducible in susceptible animals by immunization with myelin antigens mixed with a suitable adjuvant (2). The leukocytes, in particular T helper (Th) cells, play a prominent role in the pathogenesis of MS and EAE (2,3). After an antigenic stimulation, naïve Th cells differentiate into several subsets including Th1, Th2, Th17, and regulatory T (*T-reg*) cells which secrete distinct cytokine profiles. The Th1 and Th17 cells were thought to be responsible for the demyelination in MS and EAE (3,4), whereas Th2 and *T-reg* cells have been shown to mostly prominent in the resolution stages of the disease (4,5). In our previous studies, higher levels of a Th17-related chemokine (CCL20) and lower levels of a Th2/*T-reg*-related chemokine (CCL22) have been observed in patients with MS (6,7).

IL-33 is a new member of the IL-1 cytokine family that exerts its effects by binding to a receptor that contains ST2L (or ST2) and IL-1R accessory proteins (IL-1RAcP). ST2 is expressed by several leukocytes including Th2 cells, mast cells, basophils, macrophages, dendritic cells, $CD8^+$ T cells, and B cells (8). IL-33 stimulates the production of pro-inflammatory cytokines in mast cells and Th2 lymphocytes (9), induces the chemotaxis of Th2 cells (10), enhances the activation of eosinophil, basophil and natural killer cells (11,12), increases both Th1- and Th2-related responses (12), prompts IFN-γ synthesis by iNKT and NK cells, and increases the number of iNKT cells in the spleen (12,13). Although IL-33 was initially identified as a Th2 cytokine, it can also enhance Th1/Th17-related immune responses (14).

IL-33 is constitutively expressed in the nuclei of both endothelial cells and epithelial cells (15). IL-33 is also expressed by some innate immune cells such as macrophages and dendritic cells (8). Several other cell types such as fibroblasts, cardiomyocytes, keratinocytes, and adipocytes have also been reported as major producers of IL-33 (16). Interestingly, the highest expression of IL-33 in mice are found in the brain and spinal cord (17) which shows that IL-33 may have particular local effects in the CNS, in addition to its roles in the immune system. The expression of IL-33 in the CNS increases in response to inflammatory inducers and Astrocytes also express both ST2L and IL-1RAcP (18). Elevated levels of IL-33 have also been demonstrated in the periphery and CNS of MS patients, implicating that IL-33 may participate in the pathogenesis of MS (19). IL-33 induces the production of IL-6, IL-13, and MCP-1 in CNS glial cells, and this induction was increased by IL-33-stimulated mast cells (20). IL-33 may also contribute to the pathogenesis of chronic autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, systemic sclerosis, and systemic lupus erythematosus (21). Therefore, IL-33 may have potent pro-inflammatory properties.

IL-27 is a heterodimeric cytokine consisting of EBI-3 and P28 subunits that exerts its effects through binding to a receptor that consists of WSX-1 and gp130 (22). IL-27 receptor (IL-27R) is expressed by some immune and other non-immune cells including T cells, B cells, NK cells, monocytes, dendritic cells, mast cells, endothelial cells, hepatocytes, and neurons, (22). Initially, IL-27 was described as a pro-inflammatory cytokine produced by antigen presenting cells (APC) that play a role in the early phase of Th1 cells differentiation (23). Subsequent investigations demonstrated the anti-

inflammatory effects of IL-27. Different studies, by using infectious and autoimmune models, have demonstrated that mice lacking the WSX-1, develop severe pathological inflammation of both Th1 and Th2 responses (22). The increased Th17-assocaited CNS inflammation has been also demonstrated in *Toxoplasma gondii* infected WSX-$1^{-/-}$ mice (22). Interestingly, it has been also reported that the IL-27R-deficient mice are very susceptible to EAE and have elevated numbers of Th17 cells (24).

It has been also demonstrated that P28 may have independent activities of EBI3 via binding to an IL-6 α-receptor (IL-6Rα) (25,26). An alternative secreted complex may also be produced by p28 and the soluble cytokine receptor cytokine-like factor 1 (CLF) (25). Like IL-27, P28/CLF is produced by dendritic cells and induces NK cells to secrete IFN-γ, inhibits CD4 T cell proliferation, induces IL-17 and IL-10 secretion, enhances B cell proliferation and promotes plasma cell differentiation (26,27).

Several genetic and environmental factors have been implicated in MS development. Among the nutritional components related to MS, vitamin D status has been more frequently studied (28,29). Low levels of vitamin D are common in MS patients and low sun exposure can also increase the probability of MS, especially in a young age (30,31). Moreover, low levels of vitamin D have been associated with higher relapses of MS (32). Vitamin D also induces changes in the gene expression profile of the immune cells derived from patients with MS (33).

It should be noted that a long-period treatment with anti-inflammatory drugs may have some serious side effects. The use of other components for the treatment of inflammatory diseases may be more effective and, by a large portion, reduce the side effects. It has been reported that vitamin D has broad anti-inflammatory and immunomodulatory effects. Therefore, the aim of this study was to examine the effects of vitamin D on the expression of IL-27 and IL-33 in the CNS of EAE mice and clinical symptoms of disease in C57BL/6 mice.

MATERIALS AND METHODS

Mice. Females, 6 to 8 weeks old, C57BL/6 (Pasteur Institute, Tehran, Iran) were used in this study. The mice were maintained in a temperature-controlled environment with a 12-hour light/12-hour dark cycle and were administered standard laboratory food and water ad libitum. All mice were housed in a room where the testing procedure was performed as to minimize any stress responses potentially triggered by novel environmental cues. All experiments were conducted on-site at the Kerman University of Medical Sciences and were performed in accordance with the guidelines of the Medical School`s Ethics Committee on Animal Experimentation.

Induction and Treatment of EAE. The EAE was induced as previously explained (34). Briefly, the C57BL/6 mice were injected, subcutaneously, (s.c.) on day 0 with 400 μg of $MOG_{35–55}$ peptide emulsified in complete Freund's adjuvant containing 5 mg/ml of M. tuberculosis at two sites in the flank. The mice received two additional intra-peritoneal (i.p) injections of 250 ng of pertussis toxin on day 0 and 48 hours post immunization. The mice were weighed and evaluated daily for clinical symptoms of the disease. The disease was scored based on the following criteria: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, paralysis of one hind limb; 4, paralysis of two hind limbs; 5, forelimb and hind limb paralysis; 6, dead (Takeuchi C et al., 2013). Paralyzed mice were given easy access to food and water.

Planning of Research. The mice were divided into 4 groups (6 mice in each) as follows: Group I (normal control group): Mice in this group were considered healthy normal term without EAE and were only treated with PBS. Group II (EAE negative control group): Mice in this group were considered as PBS-treated EAE group without receiving vitamin D. Group III (EAE negative control group): The mice with EAE enrolled into this group and treated only with olive oil (as vitamin D vehicle). Mice in this group were also considered as olive oil-treated EAE group Group IV (vitamin D-treated EAE group): The mice with EAE enrolled into this group and treated with 200 ng of vitamin D.

The 1, 25 dihydroxyvitamin D3 (1,25(OH)2D3, C27H44O3, MW 416.6) was purchased from Osveh company (Tehran, Iran). The mice were immunized on day 0 with injection of an emulsion of MOG peptide and complete Freund adjuvant containing mycobacterium tuberculosis to induce EAE. The mice were intra peritoneal (i.p) treated with 100 µl vehicle (olive oil) or PBS in control groups and 200 ng vitamin D in same volume of olive oil (every two days) from day +3 to +30 in treatment groups. The EAE clinical scores and body weights were evaluated till day 30. At day 31 all the mice were scarified, their spinal cord and brain removed for further analysis.

Histology. Brains and spinal cords were removed and fixed in 4% buffered formalin fixative overnight. Five-micrometer thick transverse sections were prepared from cervical region of the spinal cord (five sections per mouse). Paraffin wax embedded sections were stained with hematoxylin and eosin to examine the inflammation. Signs of inflammation in the anterior, posterior, and two lateral columns (four quadrants) of the spinal cord sections were scored under a light microscope, as previously described (35). Briefly, each quadrant displaying the infiltration of mononuclear cells was assigned a score of one inflammation point. The pathological score for each group was expressed as percentage over the total number of quadrants examined.

Real-Time PCR. The expression of the IL-27 and IL-33 mRNA in the spinal cord was measured by RT-PCR. The β-actin gene used as internal control. The used primers have been demonstrated in Table 1. Total RNA was extracted from spinal cord using Trizol Reagent (Invitrogen, Carlsbad, CA). The purity of the extracted RNA was determined by electrophoresis on an ethidium bromide pre-treated agarose gel along with measuring absorption by spectrophotometer and calculation of 260/280 ratio. The RNA was converted to cDNA using a cDNA synthesis kit (Bionner, Korea) with both oligo (dT) and random hexamer primers. The process of reverse transcription was performed by the following protocol: 70°C for 10 min (without reverse transcription enzyme), 20°C for 1 min (cooling step), addition of reverse transcription enzyme, 42°C for 60 min, and the protocol was completed by final step at 95°C for 10 min to terminate the activation of the reverse transcription enzyme.

Real-time PCR was performed using a SYBR green master mix (Bionner, Korea), combined with 200 ng of template cDNA with the appropriate primers (Table 1) in a Bio-Rad CFX96 system (Bio-Rad Company, USA) using the following program: 1 cycle of 95°C for 15 min, 40 cycles of 95°C for 30 s and 60°C for 30 s and finally 72°C for 30 s. Primers were synthesized by the Bionner Company (Korea). Real-Time PCR was carried out in triplicate and the β-actin was applied as a housekeeping gene for the normalization of the amplified signals of the target genes. The sequences of the used primers are shown in Table 1. The quantity of cytokines mRNA in the spinal cord, expressed as units relative to the amount of β-actin mRNA. The dissociation stages,

melting curves and quantitative analyses of the data were performed using a CFX manager software version 1.1.308.111 (Bio-Rad, USA).

Statistical Analysis. Data are presented as mean ± SEM. Statistical analysis was performed using Kruskal-Wallis and Mann-Whitney U tests, as seen fit. P values less than 0.05 were considered statistically significant.

Table 1. The used primers for the gene expression of IL-27 and IL-33 in the spinal cord.

Gene	Primer
IL-33	Forward: ATCACGGCAGAATCATCGAG
	Reverse: CTTATGGTGAGGCCAGAACG
IL-27 (P28)	Forward: ATCTCGATTGCCAGGAGTGA
	Reverse: GTGGTAGCGAGGAAGCAGAGT
IL-27 (EBI3)	Forward: GCCATGCTTCTCGGTATCC
	Reverse: GAGCCTGTAAGTGGCAATGA
β-Actin	Forward: AGAGGGAAATCGTGCGTGAC
	Reverse: CAATAGTGATGACCTGGCCGT

RESULTS

The Effect of Vitamin D on the Clinical Symptoms of EAE. The clinical scores of EAE in vitamin D-treated EAE mice and control groups are demonstrated in Figure 1. The PBS- or olive oil-treated EAE mice showed clinical symptoms of EAE at days 10 and 9, respectively. The vitamin D-treated EAE group exhibited the clinical symptoms at day 13. The maximum mean clinical score (MMCS) was 5.0 ± 0.00 for PBS-treated EAE mice, 4.4 ± 0.24 for olive oil-treated EAE and 2.2 ± 0.66 for EAE mice treated with vitamin D. The MMCS was significantly lower in vitamin D-treated EAE groups compared with PBS- or olive-treated EAE mice ($p<0.008$ and $p<0.02$, respectively; by using Mann-Whitney U test). Accordingly, vitamin D significantly decreased the severity of EAE.

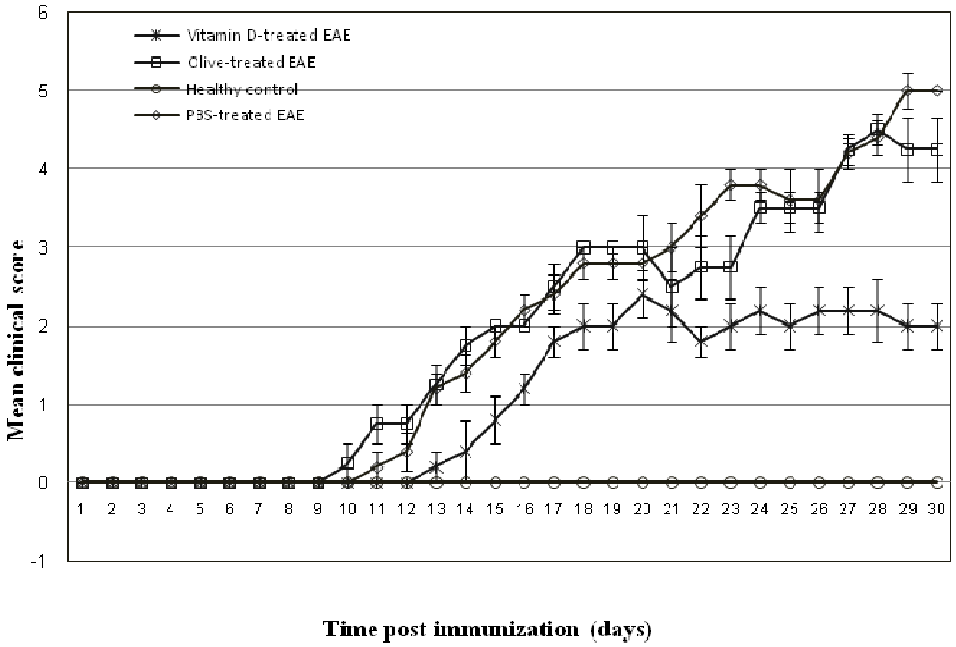

Figure 1. Comparison of the clinical scores of the EAE between vitamin D-treated and the control groups. The maximum mean clinical score was significantly lower in vitamin D-treated EAE groups compared with PBS- or olive treated EAE mice ($p<0.008$ and $p<0.02$, respectively; by using Mann-Whitney U test)

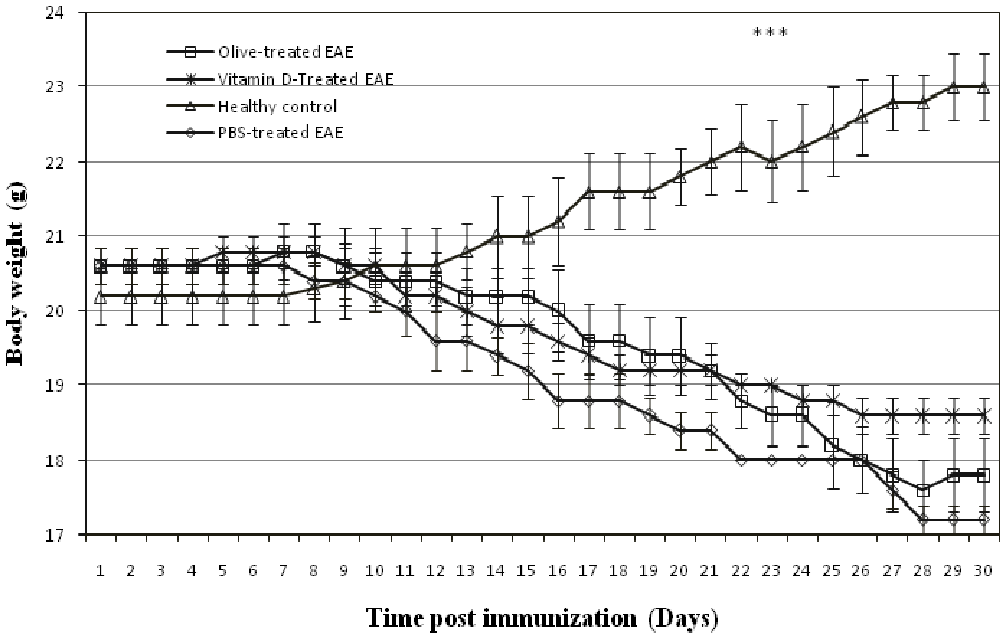

Figure 2. Comparison of the body weight between vitamin D-treated EAE and control groups. The mean body weight in PBS-treated EAE was significantly lower than that observed in the normal control group, 15 days post immunization (***; $p<0.02$ at 15-17 days and $p<0.008$ at 17-30 days, by using Mann-Whitney U test). The total body weight in the olive oil-treated EAE mice was also significantly lower than the healthy control group`s, 17 days post immunization ($p<0.03$ at 17-19 days, $p<0.01$ at 20 day and $p<0.008$ at 21-30 days, by Mann-Whitney U test). The body weight in the vitamin D-treated group was significantly higher than that of PBS-treated EAE mice ($p<0.008$ at 22-23 days, $p<0.03$ at 24-25 days, $p<0.05$ at 27 day and $p<0.01$ at 28-30 days, by using Mann-Whitney U test)

The Effects of Vitamin D on Body Weight. The effects of the treatment with vitamin D on the body weight have been demonstrated in Figure 2. The mean body weight in the PBS-treated EAE mice was significantly lower than that measured in the normal control group, 15 days post MOG immunization ($p<0.04$ at 15-17 days and $p<0.008$ at 17-30 days, by using Mann-Whitney U test).

The mean body weight in the olive oil-treated EAE mice was also significantly lower than that of the healthy control group`s, 17 days post MOG immunization ($p<0.03$ at 17-19 days, $p<0.01$ at 20 day and $p<0.008$ at 21-30 days, by Mann-Whitney U test). A similar pattern of body weight loss was observed in PBS-treated.

EAE group, compared with the normal control group during 17-30 days post MOG immunization ($p<0.02$ at 17 day, $p<0.008$ at 18-30 days, by using Mann-Whitney U test). However, the mean body weight in the vitamin D-treated group was significantly higher when compared to PBS-treated EAE mice ($p<0.008$ at 22-23 days, $p<0.03$ at 24-25 days, $p<0.05$ at 27 day and $p<0.01$ at 28-30 days, by using Mann-Whitney U test).

The differences in the mean body weight of vitamin D-treated group and olive oil-treated EAE mice were not statistically significant during the post immunization period. Also, no significant differences were observed between vitamin D-treated EAE group and olive oil-treated EAE mice regarding the mean body weight during the post immunization period.

The Effect of Vitamin D on the Infiltration of Leukocytes into CNS. The influences of vitamin D on the infiltration of inflammatory cells into the CNS have been demonstrated in Figures 3 and 4. As demonstrated in Figure 3, the PBS- or olive oil treated EAE mice developed serious inflammation in their CNS, whereas treatment with vitamin D significantly diminished the infiltration events.

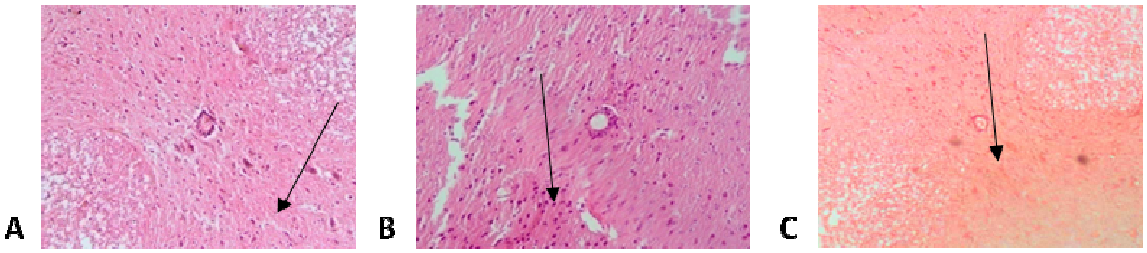

Figure 3. Comparison of the inflammatory cell infiltration in the CNS between vitamin D-treated EAE mice and control groups.

Treatment with vitamin D inhibits the inflammatory cell infiltration in the CNS. The spinal cord section from vitamin D treated EAE mice (A), PBS-treated EAE mice (B) or healthy control mice (C).

The mean pathological scores for vitamin D (23.4 ± 1.02) were significantly lower than that those observed in PBS-treated EAE group or olive oil-treated EAE mice (35.8 ± 0.58 and 35.6 ± 0.67, respectively; $p<0.008$, by using Mann-Whitney U test) (Figure 4). These results suggest that vitamin D inhibited CNS inflammation in EAE mice.

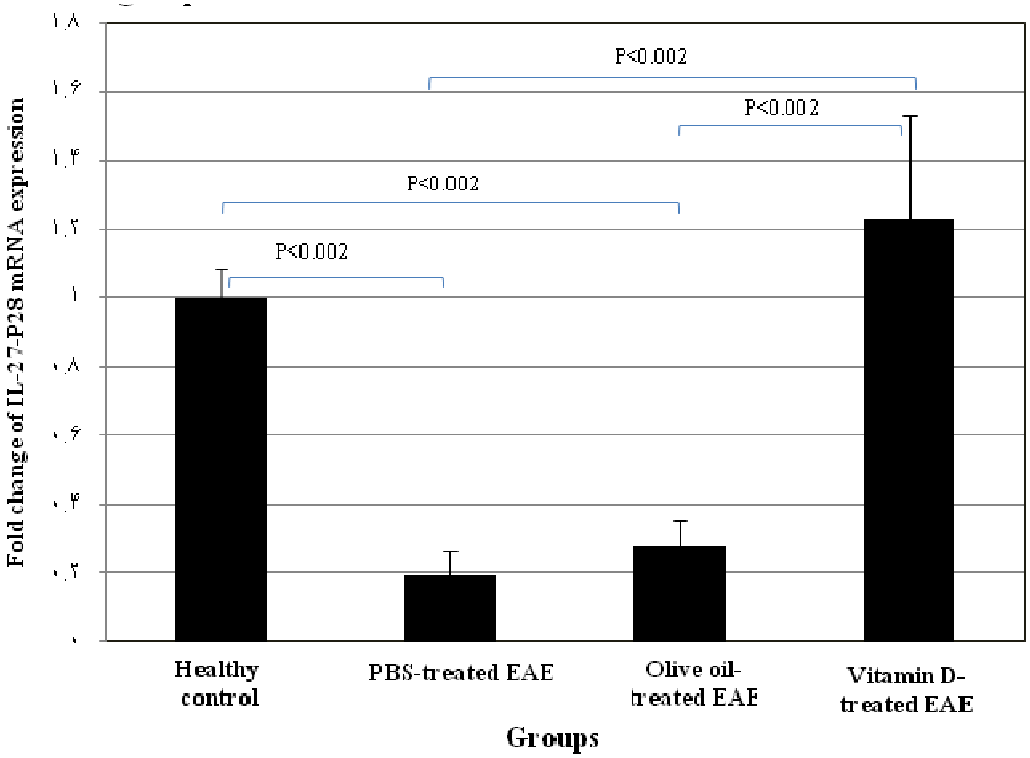

Figure 5. Comparison of the expression of IL-27 P28 mRNA in the spinal cord between vitamin D-treated EAE and control groups. In the PBS- or olive oil-treated EAE mice, the expression of IL-27 P28 mRNA was significantly lower than that of the healthy group ($p<0.002$, by using Mann-Whitney U test). In vitamin D-treated EAE group, the expression of IL-27 P28 mRNA was significantly higher compared with the PBS- or olive oil-treated EAE mice ($p<0.002$, by using Mann-Whitney U test).

Effect of Vitamin D on the Expression of IL-27 in the Spinal Cord. We analyzed the expression of IL-27 P28 mRNA in the CNS by quantitative real time RT-PCR (Figure 5). The expression of IL-27 P28 mRNA was 1.00 ± 0.08 in the healthy normal group, 0.19 ± 0.07 in PBS-treated EAE group, 0.27 ± 0.07 in the olive oil-treated EAE mice and 1.23 ± 0.30 in vitamin D-treated EAE group. In the PBS- or olive oil-treated EAE mice, the expression of IL-27 P28 mRNA was significantly lower than that observed in the healthy control group ($p<0.002$, by using Mann-Whitney U test). In tamin Dvi-treated EAE group, the expression of IL-27 P28 mRNA was significantly higher, compared with the PBS- or olive oil-treated EAE mice ($p<0.002$, by using Mann-Whitney U test). No significant difference was observed between vitamin D-treated EAE mice and the healthy control group regarding the expression of P28, although this parameter was found to be higher in vitamin D-treated EAE group. The difference in the expression of P28 mRNA between PBS- and olive oil-treated EAE groups was also not significant.

The expression of IL-27 EBI3 mRNA was 0.99 ± 0.14 in the healthy control group, 0.72 ± 0.10 in PBS-treated EAE group, 0.70 ± 0.10 in olive oil-treated EAE mice and 1.64 ± 0.42 in vitamin D-treated EAE mice (Figure 6). In both PBS- and olive oi-treated EAE groups, the expression of IL-27 EBI3 mRNA was lower than that observed in healthy normal group, but the differences were not statistically significant. In vitamin D-treated

EAE group the expression of IL-27 EBI3 was significantly higher compared with olive oil-treated EAE mice ($p<0.04$, by using Mann-Whitney U test). Similarly, in vitamin D-treated EAE group the expression of IL-27 EBI3 was higher in comparison with PBS-treated EAE mice ($p<0.06$, by using Mann-Whitney U test).

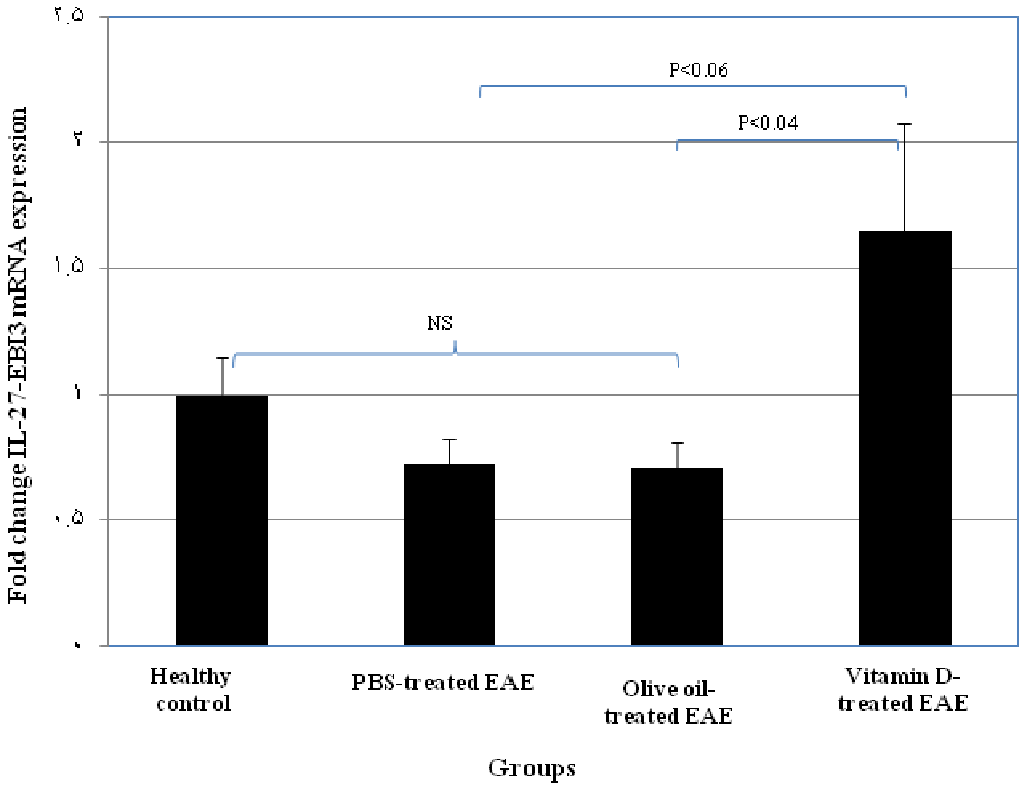

Figure 6. Comparison of the expression of IL-27 EBI3 mRNA in the spinal cord between vitamin D-treated EAE and control groups. Statical analysis by using Kruskal-Wallis test showed that in both PBS- and olive oil-treated EAE groups, the expression of IL-27 EBI3 mRNA was lower than that observed in healthy normal group but the differences were not statistically significant (NS). In the vitamin D-treated EAE group the expression of IL-27 EBI3 was higher when compared to PBS- or olive oil-treated EAE mice ($p<0.06$ and $p<0.04$, respectively; by using Mann-Whitney U test).

The IL-27 EBI3 mRNA was similarly expressed in PBS- and olive oil-treated EAE groups. No significant difference was also observed between vitamin D-treated EAE and healthy groups regarding the expression of IL-27 EBI3, although this parameter was found to be higher in vitamin D-treated EAE group, yet again.

Effect of Vitamin D on the Expression of IL-33 in the Spinal Cords. The expression of IL-33 mRNA was significantly higher in the spinal cords of PBS-treated EAE mice (2.15 ± 0.44) or olive-treated EAE group (2.91 ± 0.70) when compared with normal control mice (0.99 ± 0.27; $p<0.05$ and $p<0.02$, respectively; by using Mann-Whitney U test) (Figure 7). The effects of vitamin D on the expression of the IL-33 mRNA have also been demonstrated in Fig 6. The expression of IL-33 mRNA in vitamin D-treated EAE mice (0.91 ± 0.28) was significantly lower compared to PBS-or olive oil-treated EAE mice ($p<0.04$ and $p<0.02$, respectively; by using Mann-Whitney U test). No

significant difference was observed between vitamin D-treated EAE mice and the healthy control group regarding the expression of IL-33 mRNA. The difference of the expression of IL-33 mRNA between PBS- and olive oil-treated EAE mice was also not significant.

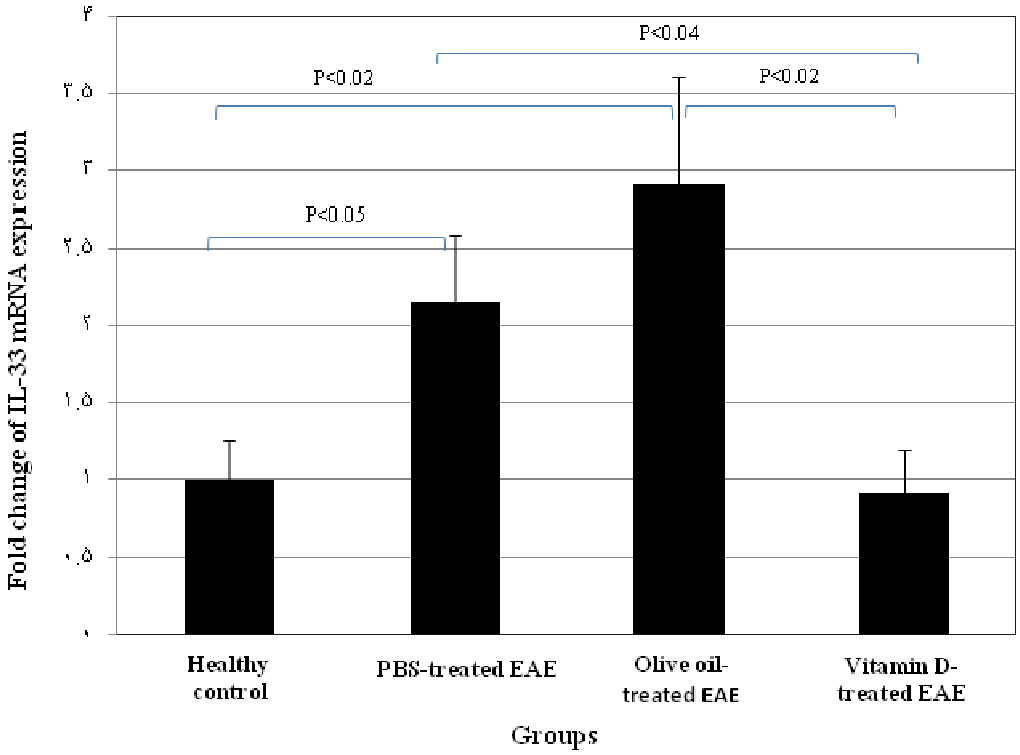

Figure 7. Comparison of the expression of IL-33 mRNA in the spinal cord between vitamin D-treated EAE and control groups. The expression of IL-33 mRNA was significantly higher in the spinal cords of PBS- or olive-treated EAE group as compared with the healthy mice ($p<0.05$ and $p<0.02$, respectively; by using Mann-Whitney U test). The expression of IL-33 mRNA in vitamin D-treated EAE mice was significantly lower compared to PBS- or olive oil-treated EAE mice ($p<0.04$ and $p<0.02$, respectively; by using Mann-Whitney U test).

DISCUSSION

The results of the present study showed that the treatment of EAE mice with vitamin D inhibits the development of EAE. The clinical symptoms of EAE appeared in PBS- or olive oil-treated EAE mice earlier in the study, in comparison with vitamin D-treated mice. The clinical scores of EAE were also significantly higher in PBS- or olive oil-treated EAE mice when compared with vitamin D-treated EAE group. These observations represent the profitable properties of vitamin D on EAE disease. A higher severity of EAE has been also been reported in nutritional vitamin D limitation (36) whereas administration of vitamin D to EAE mice confers disease protection through effects on cytokine synthesis and apoptosis of inflammatory cells (37,38). Some effects of vitamin D on EAE have been reported to be dependent of IL-10 (39). Several epidemiology studies have also reported an association between vitamin D deficiency and the incidence and/or severity of MS (40). Indeed, women with higher levels vitamin

D intakes (used as supplementation) had a 40% reduction in their risk of developing MS (41). It has also been reported that vitamin D deficiency is common in patients with MS (42). In another study, treatment with vitamin D decreased the inflammatory reactions in MS, as revealed by an anti-inflammatory cytokine profile (43). These observations are consistent with our findings.

The inhibitory effects of vitamin D on inflammatory reactions may attribute, in part, to its capability to modulate the phenotype and function of dendritic cells (DC) (44) and the inhibition of the maturation of monocyte-derived DCs (45). It should be noted that the DCs are divided into two groups, based on their origin, of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) that express different types of cytokines and chemokines and appear to exert complementary effects on T-cell responses. It has been demonstrated that vitamin D signaling can act as a natural inhibitory mechanism on both cDCs and pDCs (46). It has also been recently reported that vitamin D is able to promote the generation of tolerogenic mature dendritic cells (mDCs) that suppresses the proliferation and activation of autoreactive T cells (47).

As previously mentioned, MS is a Th1/Th17 mediated autoimmune disease (48,49). It has been demonstrated that vitamin D impairs the capacity of murine and human pDCs to induce T-cell proliferation and secretion of the Th1-related cytokines (46). It has also been reported that vitamin D preferentially inhibits the Th1 cells and enhances Th2 cells- type cytokines production (50). These observations represent that vitamin D promotes a shift from Th1- to Th2 responses and thus, may limit Th1-associated immune responses.

In experimental models of colitis, the treatment with vitamin D decreases the expression of IL-17 (51) but vitamin D deficiency, as a result of CYP27b1 gene destruction, causes higher levels of this cytokine (52). Thus, it is possible for some anti-inflammatory effects of vitamin D to perform through controlling of Th17 cells. It has been also reported that vitamin D actively regulates cytokine production by $CD8^+$ T cells (53). The regulatory effects of the vitamin D on B cell proliferation and immunoglobulin production have also been reported (54).

In accordance with reducing the clinical scores, histological results represent that vitamin D significantly reduced the infiltration of the inflammatory cells into the spinal cord. The infiltration of leukocytes into the CNS is an important step in the pathogenesis of MS that controls by chemokines (55). Chemokines are a group of small polypeptide, which attract various types of leukocytes to inflammation sites and play an important role in their immunity as well as in inflammatory and autoimmune disease (56).

It has been demonstrated that the vitamin D suppresses the monocytes and macrophages migration through the inhibition of RANTES and MCP-1 production (57,58). The RANTES promoting the leukocyte infiltration and the elevated levels of RANTES have been associated with many inflammatory disorders. MCP-1 is responsible for the migration of monocytes and macrophages into the inflammatory sites**.** It has also been reported that vitamin D inhibits the production of MCP-1 (58). Accordingly, the administration of vitamin D postponed the onset of EAE and significantly suppressed both the clinical and the pathological severity of the disease.

The results of the present study also demonstrated that the expression of IL-33 mRNA was significantly higher in the spinal cords of PBS- or olive oil- treated EAE groups. These observations imply that IL-33 may play a prominent role in the pathogenesis of EAE. Elevated levels of IL-33 have been linked to some inflammatory diseases such as

rheumatoid arthritis and allergic inflammation (59,60). Although, elevated expression of the IL-33 mRNA has been demonstrated in the brain and spinal cord of mice (17), but there are controversial studies regarding its role on the EAE (61).

It has been reported that the Th1 and Th17 cells play an important role during development of the EAE (3). It has been also demonstrated that IL-33 drives Th1/Th17 responses in some experimental models of immune disorders (12). The antigen-stimulated lymphocytes from IL-33-treated mice, were produced more IL-17 and IFN-γ (62). Accordingly, IL-33 may involve in the pathogenesis of the EAE, through the increasing of Th17 and Th1 cell functions.

Furthermore, we have observed that the treatment with vitamin D suppresses the expression of IL-33 in the spinal cord of EAE mice. Consistent with our results the amelioration of EAE has been reported in the anti-IL-33-treated EAE mice (62). The amelioration of the EAE in anti-IL-33-treated EAE mice has been attributed to the reduction of IL-17 and IFN-γ production (62). The attenuation of EAE severity by vitamin D may be eventually linked to the suppression of the both Th1 and Th17 responses. Accordingly, the decreasing effects of vitamin D on the IL-33 mRNA may results to the modulation of the both Th1 and Th17 responses.

The *T-reg* cells are other important subsets of $CD4^+$ T cells that have a main role in maintaining of immune tolerance. Mice or humans lacking *T-reg* cells, suffered from severe immune disorders (63). The *T-reg* cells exert their effects through several mechanisms including secretion of inhibitory cytokines such as IL-10 and TGF-β (64). Previous studies in the EAE model demonstrated that adoptive transfer of *T-reg* cells conferred significant protection against EAE which was linked with normal Th1 cells activation, increased production of Th2 cytokines and decreased leukocytes infiltration into the CNS (65)**.** Moreover, the drugs which enhancing the *T-reg* differentiation or inhibiting Th17 development could ameliorate EAE (66). It has been reported that the neutralization of the IL-33 by using monoclonal antibody increases the expression of IL-10 and TGF-β in the CNS of mice with EAE (62). Accordingly, the reducing effects of vitamin D on the IL-33 mRNA in EAE may results to the higher *T-reg* cells-related responses. It has been demonstrated that vitamin D, alone or in conjunction with glucocorticoids, strongly stimulated the differentiation of *T-reg* cells (67-68).

The results of the present study also demonstrated the lower expression of both subunits of IL-27 including P28 and EBI3 mRNA in PBS- or olive oil-treated EAE mice. These observations represent that the down-regulation of the expression IL-27 mRNA may contribute a role in the development of EAE. In accordance with our results, it has been reported that the inhibition of the interaction of IL-27 with its receptor results to the more severe EAE (69). Moreover, IL-27 suppresses the generation of Th17 (69). Furthermore, it has been also reported that the IL-27 can suppresses the inflammatory effects of IL-17A and cause to the partial amelioration in clinical symptoms and development of MS disease (70). On the other hand, IL-27, by promotion of IL-10 secretion, can inhibit the inflammatory response (71). The diminished expression of IL-27 in EAE mice may result in the elevation responses of Th17 cells. These observations encourage more studies on the anti-inflammatory effects IL-27 and therapeutic potential of this cytokine in patients with MS.

Our results also demonstrated the treatment of EAE mice with vitamin D improve the expression of IL-27 mRNA. In vitamin D -treated EAE group the expression of IL-27 mRNA was higher in comparison to PBS-treated EAE mice. The exact mechanisms of vitamin D on IL-27 production remain to be clear in future studies. However, the

vitamin D may directly and/or indirectly influence the IL-27 production. For example, it has been reported that PGE2 (a production of COX-2) significantly inhibits LPS-induced IL-27 production by monocytic cell line (72). On the other hand, the suppressive effects of the vitamin D on the COX-2 have been demonstrated (73). Accordingly, the vitamin D may enhance the expression of IL-27 through the suppression of COX-2.

It should be noted that the absence a vitamin D-treated healthy group may be consider a limitation of our study. The aim of this study was to evaluate the effects of vitamin D on a number of parameters in a model of EAE. These results may be extended to the treatment of MS patients by vitamin D. Therefore, it seems that the absence of a vitamin D-treated healthy group may have had no influence on the validity and reliability of our data.

In summary, the results of the present study demonstrated alterations in the expression of IL-27 and IL-33 mRNA in EAE mice. The vitamin D -treated EAE mice exhibited mild signs of EAE, a delay in disease onset and low infiltration of the inflammatory cell into the spinal cord. Moreover, treatment with vitamin D modulates the expression of IL-27 and IL-33 mRNA in EAE mice. These observations represent that vitamin D has a significant capability to ameliorate the severity of EAE.

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