**Original Article**

1,25(OH)_2D_3 suppresses proinflammatory responses by inhibiting Th1 cell differentiation and cytokine production through the JAK/STAT pathway

Zehua Zhang1, Feifan Chen2, Jianhua Li1, Fei Luo1, Tianyong Hou1, Jianzhong Xu1, Dong Sun1

1Department of Orthopedics, Southwest Hospital, Third Military Medical University, Chongqing, China; 2Emergency Room, The First Affiliated Hospital, Zhengzhou University, Henan, China

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**Abstract:** 1,25(OH)_2D_3 is an immune modulation hormone with beneficial effects on T cell-mediated autoimmune diseases. The purpose of the present study is to investigate the direct effects of vitamin D on Bacillus Calmette Guerin (BCG)-infected CD4+ T cells in both VDR-deficient (VDR-/-) mice and wild type (WT) mice. Specifically, we aimed to investigate the effect of vitamin D on Th1 cells and elucidate the underlying molecular mechanism. Naïve CD4 T cells were purified from VDR-/- mice and WT mice to induce Th1 cells and were activated by BCG. Th1 cell differentiation and cytokine production in vitro were inhibited by 10 nM 1,25(OH)_2D_3. The JAK/STAT pathway was activated by 1,25(OH)_2D_3 addition in both VDR-/- and wild type T cells. In vivo, a vitamin D-deficiency VDR-/- and WT mouse model was established and the mice were vaccinated with BCG. An ELISA assay was performed to measure the levels of VD, IL-2, IFN-γ and TNF-β in the blood, and flow cytometry was used to analyze the proportion of Th1 and Th2 cells in the spleen. 1,25(OH)_2D_3 affected Th cells polarization by inhibiting Th1 and augmenting Th2 cell development in the vitamin D-deficiency mouse model. Moreover, 1,25(OH)_2D_3 inhibited the inflammatory infiltrates and expression of IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG. These findings suggested that 1,25(OH)_2D_3 suppressed the inflammatory response by inhibiting Th1 cell differentiation and cytokine production by the JAK/STAT pathway.

**Keywords:** 1,25(OH)_2D_3, VDR, JAK/STAT, Th1 cells, inflammatory response

**Introduction**

1,25-Dihydroxyvitamin D3 (1,25(OH)_2D_3), the active form of vitamin D, is a seco-steroid hormone primarily synthesized in human skin following stimulation of by ultraviolet (UV) radiation. Vitamin D is not only involved in mineral and skeletal homeostasis, but also plays an important role in bone metabolism and immune modulation [1, 2]. Vitamin D can also enhance the microbicidal ability of monocytes/macrophages and downregulate inflammatory cytokines produced by T lymphocytes [3].

Many types of immune cells, including circulating monocytes, macrophages, dendritic cells (DCs), and activated T cells express a nuclear vitamin D receptor (VDR). Previous studies reported that 1,25(OH)_2D_3 addition inhibited Th1 cell development and cytokine production while inducing Th2 cell expansion and increasing IL-4 production [4]. Conversely, Staeva-Vieira et al. have reported that both Th1 and Th2 cell responses were inhibited by 1,25(OH)_2D_3 [5]. The results were conflicting. However, the direct effects of vitamin D on Bacillus Calmette Guerin (BCG)-infected CD4+ T cells from VDR-deficient (VDR-/-) mice and wild type (WT) mice was unclear. Specifically, the molecular mechanism of the effect of vitamin D on Th1 cells has not been defined.

In our study, we found that both Th1 cell differentiation and relative cell cytokine (IL-2, IFN-γ and TNF-β) production were inhibited. Knockout of VDR attenuated the inhibitory effect of 1,25(OH)_2D_3 on Th1 cell differentiation and relative cell cytokine production, indicating that the inhibitory effect of 1,25(OH)_2D_3 might be mediated by VDR. 1,25(OH)_2D_3 exerts its actions...
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through VDR and activates or represses target genes by binding to vitamin D responsive elements on DNA [6].

Then we gained insight into the possible mechanism of 1,25(OH)2D3 and found that 1,25(OH)2D3 inhibited Th1 cell differentiation and cytokine production by the JAK/STAT pathway. Collective data suggested that JAK/STAT signaling pathway was involved in the regulation of a number of immune cells such as CD4 T cells, which critically orchestrate inflammatory responses [7, 8].

In vivo, 1,25(OH)2D3 was found to affect Th cell polarization by inhibiting Th1 and augmenting Th2 cell development in a vitamin D deficiency mouse model. Th1 cells secrete IL-2, IFN-γ and lymphotoxin and direct cell-mediated immune responses, while Th2 cells secrete IL-4, IL-5, IL-6 and IL-13 and mediate humoral responses [5]. 1,25(OH)2D3 may regulate the Th1/Th2 cell balance to influence the outcome of the immune response. Additionally, intra-tumoral leukocyte population infiltration had occurred, and IL-2, IFN-γ and TNF-β expression were decreased in the spleen following 1,25(OH)2D3 treatment. Thus, 1,25(OH)2D3 was an immunoregulator, and its level at sites of inflammation may have a significant effect on the initiation of the immune response.

These studies elucidated the mechanisms in the interaction between vitamin D and immune system and provide a foundation for targeting Th1 immunity by 1,25(OH)2D3 analogs.

Materials and methods

Animals

VDR-deficient (VDR/-) mice on a B6/CBA genetic background were backcrossed more than 8 times to C57BL/6J mice and maintained in our colony for more than 7 years [9]. Vitamin D deficiency was induced in C57B/6 mice (VDR-/- and WT mice) by keeping them in a UV-free environment and by feeding them a vitamin D-depleted diet (< 200 IU/kg vitamin D) containing 20% w/w lactose, 2% w/w calcium, and 1.25% w/w phosphorus (Ssniff Bio-services, Uden, The Netherlands). Three month later, blood levels of 1,25(OH)2D3 were measured. Mice deficient in 1,25(OH)2D3 were chosen for further study. The normal group was maintained in a UV-free environment and by feeding with a vitamin D-abundant diet. The mice received an intravenous injection in the tail, with a dose of 5 × 106 CFU BCG in a volume of 0.1 ml. The mice were separated into four groups: VDR/- + Saline, VDR/- + 1,25(OH)2D3, WT + Saline and WT + 1,25(OH)2D3. All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of Southwest Hospital, Third Military Medical University.

Preparation of naïve CD4+ T cells

Naïve CD4+ T cells were obtained from the spleens of VDR-deficient (VDR-) mice and WT mice by physically mincing the tissue through a 40-μm nylon cell strainer and subse-quent culturing of cells in RPMI-1640 in the presence of 10% FBS. A CD4+ T cell isolation kit (MiltenyiBiotec) was used to isolate the CD4+ T cells from mouse splenocytes following the manufacturer’s protocol. The cell proportion of purified naïve CD4+ T cells before and after isolation was tested by flow cytometry.

Preparation of Th1 cells

The antigen presenting cells from the spleen of VDR-deficient (VDR-) mice and WT mice were isolated as previously described [10]. Briefly, mononuclear lymphocytes from spleen were incubated with CD4, CD8, antibody, and complement to eliminate the T cells. Then, the cells were treated with mitomycin (Sigma, St. Louis, MO) and cultured in RPMI-1640 in the presence of 10% FBS. A CD4+ T cell isolation kit (MiltenyiBiotec) was used to isolate the CD4+ T cells from mouse splenocytes following the manufacturer’s protocol. The cell proportion of purified naïve CD4+ T cells before and after isolation was tested by flow cytometry.

Hematoxylin and eosin (HE) staining

Spleen sections were stained with hematoxylin for 2 min and then eosin for 30 s. Then, the slides were washed, dehydrated and sealed with Entellan (Merck). Images of the spleen from multiple sections in each mouse were captured using identical parameters.
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**Table 1.** Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-F</td>
<td>AGGTCCGGTGTAACCGGATTG</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>CCTCTGGAGGCTCGTGGCG</td>
</tr>
<tr>
<td>VDR-F</td>
<td>GAATGTGGCTGGATCTGG</td>
</tr>
<tr>
<td>VDR-R</td>
<td>ATGCCGGAACTCTCATTGAAG</td>
</tr>
<tr>
<td>IFN-γ-F</td>
<td>ACGAAGGCGAAAAGGT</td>
</tr>
<tr>
<td>IFN-γ-R</td>
<td>TGGTGGACACTCGGATGA</td>
</tr>
<tr>
<td>TNF-β-F</td>
<td>CCCATCACCTCCAGAAG</td>
</tr>
<tr>
<td>TNF-β-R</td>
<td>CATGTCGGAAAGGCGAAG</td>
</tr>
<tr>
<td>IL-2-F</td>
<td>TGACCGGAGAATACAG</td>
</tr>
<tr>
<td>IL-2-R</td>
<td>GTCCAAGTTCATCTTCCAGC</td>
</tr>
</tbody>
</table>

**Immunohistochemistry assay**

Spleen sections were fixed in neutral buffered formalin and embedded in paraffin. Sections measuring 4 μm in thickness were transferred to pre-treated glass slides, deparaffinized, hydrated, blocked for endogenous peroxidase using 3% H₂O₂/H₂O, and subsequently subjected to microwave epitope. The section were incubated with primary antibodies specific for IL-2, IFN-γ and TNF-β for 30 min at room temperature. Negative control sections were incubated with diluted normal human serum instead of primary antibodies. A universal biotinylated secondary antibody (DAKO) was applied, followed by incubation with peroxidase substrate 3, 3-diaminobenzidine (DAB) (Sigma-Aldrich) and counterstaining with Harris’ hematoxylin.

**Western blot**

Western blot was performed as previously described [8]. Briefly, cells were lysed in lysis buffer containing 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.02% NaN₃, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% Triton. Protein samples were subjected to 4-12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked for 1 h in TBST containing 2% BSA, and then incubated with antibodies against GAPDH (Santa Cruz, 1:1000), VDR (CST, 1:1000), IL-2 (CST, 1:1000), IFN-γ (CST, 1:1000), TNF-β (CST, 1:1000), P-JAK (Santa Cruz, 1:1000), JAK (Santa Cruz, 1:1000), P-STAT (Santa Cruz, 1:1000), STAT (Santa Cruz, 1:1000) and HRP-conjugated goat anti-rabbit secondary antibodies (Promab, 1:1000). Protein bands were detected by a chemiluminescence kit (Santa Cruz Biotechnology).

**Cell viability (MTT) assay**

Briefly, the cells (5000/well) were incubated in triplicate in a 96-well plate in the presence of various concentrations of 1,25(OH)₂D₃ (1, 10, or 100 nM) or vehicle at 37°C and 5% CO₂ in a humidified chamber for 24 h, 48 h, or 72 h. At different time points, 20 μL of MTT solution was added to each well, and cells were incubated at 37°C in a humidified chamber for 4 h. After 4 h, the supernatant was removed, and the colored formazan crystal produced from MTT was dissolved. The plates were then analyzed by ELISA, and absorbance at 570 nm was measured.

**RNA extraction and quantitative reverse transcription PCR (Q-PCR)**

Total RNA was extracted from cells using Trizol Reagent (Life technologies) following the manufacturer’s protocols. For Q-PCR analysis, SYBR green PCR Mix (iTAP, Bio-Rad) was used and samples were run in an Applied Biosystems (ABI) step-one plus sequence detection system (Applied Biosystems, Foster City, CA). Fold change was calculated from the ΔΔCT values with the formula 2-ΔΔCT. The primers used in this study are listed in Table 1.

**Statistical analysis**

Statistical analyses were performed using SPSS 19.0 software. All results are presented as the mean ± S.E.M from at least three independent experiments, unless otherwise indicated. Student’s t-test was employed to assess differences between two groups. A value of P < 0.05 was considered statistically significant.

**Results**

1,25(OH)₂D₃ inhibited the Th1 cell differentiation and cytokine production in vitro

To test the effect of 1,25(OH)₂D₃ on the Th1 cell polarization process, we conducted in vitro Th differentiation experiments. Briefly, naive CD4 T cells were purified from VDR-deficient (VDR-/+) mice and wild type (WT) mice. The cell proportions of purified naive CD4+ T cells after isolation were evaluated and are shown in Figure 1A. The purified naive CD4+ T cells were activated in culture under Th1-skewing conditions in the presence of 1,25(OH)₂D₃. First, we assessed the viability of Th1 cells in the presence of 1, 10, or 100 nM 1,25(OH)₂D₃ or in the pre-
1,25(OH)₂D₃ inhibits Th1 cell differentiation and cytokine production

As shown in Figure 1B, 1,25(OH)₂D₃ stimulation affected the viability of Th1 cells in a dose-dependent manner. We chose 10 nM 1,25(OH)₂D₃ as the appropriate concentration for subsequent assays. Naive CD4+ T cells were cultured under Th1-skewing conditions in the absence or presence of 1,25(OH)₂D₃. Then, Th1 cell proportions and levels of relative cell cytokines were evaluated.

We found that the levels of IL-2, IFN-γ and TNF-β in cultural supernatants were decreased in the presence of 1,25(OH)₂D₃ in T cells of both VDR/- and wild type T cells. In addition, the knockout of VDR attenuated the inhibitory effect of 1,25(OH)₂D₃ on IL-2, IFN-γ and TNF-β production (Figure 1C-E). 1,25(OH)₂D₃ was found to inhibit both VDR/- and wild type Th1 cell differentiation, with the Th1 cell proportion significantly
1,25(OH)_{2}D_{3} inhibits Th1 cell differentiation and cytokine production

Figure 2. 1,25(OH)_{2}D_{3} inhibited Th1 cell differentiation and cytokine production by the JAK/STAT pathway. A. The mRNA levels of VDR, IL-2, IFN-γ and TNF-β in induced VDR-/- and WT CD4+ cells in the absence or presence of 1,25(OH)_{2}D_{3} were tested by Q-PCR. B. Western blot was used to analyze the protein levels of VDR, IL-2, IFN-γ and TNF-β in the absence or presence of 1,25(OH)_{2}D_{3}. C. The protein level of P-JAK, P-STAT, JAK and STAT was tested by Western blot. D. Densitometry plot of results from Figure 3B. Relative expression levels were normalized to GAPDH. Data represent the mean ± standard error (n = 3). E. Densitometry plot of results from Figure 3C. Relative expression levels were normalized to GAPDH (n = 3). Data represent three independent experiments (average and standard error of the mean of triplicate samples). NS P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

decreased after 1,25(OH)_{2}D_{3} treatment. VDR knockout attenuated the inhibitory effect of 1,25(OH)_{2}D_{3} on Th1 cell differentiation, indicating that the inhibitory effect of 1,25(OH)_{2}D_{3} might be mediated by VDR (Figure 1F).

1,25(OH)_{2}D_{3} inhibited Th1 cell differentiation and cytokine production by the JAK/STAT pathway

To gain insight into the possible mechanism of 1,25(OH)_{2}D_{3} inhibition of Th1 cell differentiation and cytokine production, we assessed the expression of VDR, IL-2, IFN-γ and TNF-β in induced CD4+ cells in the absence or presence of 1,25(OH)_{2}D_{3}. As shown in Figure 2A, 2B, both the mRNA and protein levels of IL-2, IFN-γ and TNF-β in induced CD4+ cells were decreased by 1,25(OH)_{2}D_{3} addition. The expression of VDR in VDR-/- T cells was lower than in wild type T cells. Moreover, the expression of IL-2, IFN-γ and TNF-β was increased in VDR-/- T cells compared with wild type T cells in presence of 1,25(OH)_{2}D_{3}. Intriguingly, we found that the levels of P-JAK and P-STAT were significantly enhanced by 1,25(OH)_{2}D_{3} addition in both VDR-/- and wild type T cells. Consistent with other results, knockout of VDR attenuated the effect of 1,25(OH)_{2}D_{3} on activation of JAK/STAT pathway (Figure 2C, 2E).

1,25(OH)_{2}D_{3} affected Th cell polarization by inhibiting Th1 and augmenting Th2 cell development in the vitamin D deficiency mouse model following vaccination with BCG

We established vitamin D deficiency VDR-/- and WT mouse models, and the mice were vac-
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Figure 3. 1,25(OH)₂D₃ affected Th cell polarization by inhibiting Th1 and augmenting Th2 cell development in the vitamin D deficiency mouse model following vaccination with BCG. A vitamin D deficiency VDR-/− and WT mouse model was established, and the mice were vaccinated by BCG with the addition of 1,25(OH)₂D₃ or saline. ELISA assay was performed to test the level of vitamin D (A), IL-2 (B), IFN-γ (C) and TNF-β (D) in the blood. (E, F) Flow cytometry was used to analyze the proportion of CD4+/IFN-γ+ and CD4+/IFN-γ+ T cells in the spleen of mice. *P < 0.05, **P < 0.01, ***P < 0.001.
1,25(OH)₂D₃ inhibits Th1 cell differentiation and cytokine production

Figure 4. 1,25(OH)₂D₃ inhibited inflammatory infiltrates and expression of IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG. (A) The mRNA level of VDR, IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG was tested by Q-PCR. (B) Western blot was used to analyze the protein level of VDR, IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG. (C) Densitometry plot of results from B. Relative expression levels were normalized to GAPDH. Data represent the mean ± standard error (n = 3). (D) Representative images of hematoxylin and eosin staining in spleen of vitamin D deficiency model mice following vaccination with BCG. Representative images of immunohistochemical staining for IL-2 (E), IFN-γ (F) and TNF-β (G) are shown. Scale bars, 100 µm. NS P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

As shown in Figure 3A, blood levels of vitamin D were elevated with 1,25(OH)₂D₃ addition in both VDR⁻/- and WT mice. The levels of IL-2, IFN-γ and TNF-β in the blood were measured. 1,25(OH)₂D₃ was found to lower IL-2, IFN-γ and TNF-β levels in the blood. In addition, the level of IL-2, IFN-γ and TNF-β in VDR⁻/- mice was increased compared...
with WT mice when 1, 25(OH)₂D₃ was added (Figure 3B-D). Then we assessed the proportion of Th1 and Th2 cells in the spleen of the mice. As shown in Figure 3E, the proportion of Th1 cells in the spleen was significantly decreased by 1,25(OH)₂D₃ addition in both VDR-/− and WT mice. With addition of 1,25(OH)₂D₃, the proportion of Th1 cells was restored by VDR knockout (Figure 3E). In contrast, 1,25(OH)₂D₃ addition increased the proportion of Th2 cells in the spleen in both VDR-/− and WT mice. The effect of 1,25(OH)₂D₃ addition on Th2 cells was dampened by VDR knockout (Figure 3F).

1,25(OH)₂D₃ inhibited the inflammatory infiltrates and expression of IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG

We analyzed mRNA and protein levels of IL-2, IFN-γ and TNF-β in the spleen of vitamin D-deficient mice following vaccination with BCG. As shown in Figure 4A, the mRNA level of IL-2, IFN-γ and TNF-β in the spleen were decreased with 1,25(OH)₂D₃ addition. Consistent with mRNA levels, both the VDR-/− and WT mice showed decreased protein expression of IL-2, IFN-γ and TNF-β in the spleen with 1,25(OH)₂D₃ addition. Meanwhile, the effect of 1,25(OH)₂D₃ addition on IL-2, IFN-γ and TNF-β protein expression was dampened by VDR knockout (Figure 4B, 4C).

To assessed inflammatory infiltration, we performed the hematoxylin eosin (HE) assay in the spleen of mice and found fewer intra-tumoral leukocyte populations in 1,25(OH)₂D₃ treated mice in both the VDR-/− and WT groups. However, more intra-tumoral leukocyte populations were found in VDR-/− mice compared with WT mice after 1,25(OH)₂D₃ treatment (Figure 4D). Immunohistochemical staining results showed that expression of IL-2 (Figure 4E), IFN-γ (Figure 4F) and TNF-β (Figure 4G) in the spleen was significantly decreased by 1,25(OH)₂D₃ in both the VDR-/− and WT groups. Consistent with those results in cells, the effect of 1,25(OH)₂D₃ addition on expression of IL-2, IFN-γ and TNF-β in the spleen was dampened by VDR knockout, indicating that the effect of 1,25(OH)₂D₃ in our study was mediated by binding to VDR. All in all, our findings demonstrated that 1,25(OH)₂D₃ suppressed inflammatory damage by inhibiting Th1 cell differentiation and cytokine production by the JAK/STAT pathway.

Discussion

Vitamin D deficiency is associated with many diseases, including sarcopenia, osteoarthritis, cardiovascular disease, infections, and transplant rejection. Vitamin D deficiency also increases the risk of some autoimmune diseases such as diabetes, multiple sclerosis, encephalomyelitis, inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus [11-13]. 1,25(OH)₂D₃ exerts its actions through a nuclear vitamin D receptor (VDR) and regulates the expression of various genes involved in innate and adaptive immunity [6, 14, 15]. VDR is a member of the steroid nuclear receptor super family, and together with its heterodimeric partner the retinoid X receptor, can activate or repress target genes by binding to vitamin D response elements on DNA [16, 17]. Quiescent CD4+ T cells have a low level expression of VDR which is increased by T cell activation.

Previous studies reported that 1,25(OH)₂D₃ could not induce the differentiation of hematopoietic bone marrow progenitors to monocytes/macrophages. VDR knockout mice exhibited normal immune cell subsets compared with wild type mice [18]. In an experimental inflammatory bowel disease model, VDR deficiency resulted in minor changes in the immune response to ovalbumin immunization. However, a substantial burst of IFN-γ secretion was observed in VDR deficiency [19]. In the current study, 1,25(OH)₂D₃ was found to inhibit both VDR-/− and wild type Th1 cell differentiation and production of relative cell cytokines (IL-2, IFN-γ and TNF-β). In vitro, VDR knockout attenuated the inhibitory effect of 1,25(OH)₂D₃. We also found that the level of P-JAK and P-STAT was significantly enhanced by 1,25(OH)₂D₃ addition in both VDR-/− and wild type T cells. The knockout of VDR attenuated the effect of 1,25(OH)₂D₃ on activation of the JAK/STAT pathway. In vitro, the proportion of Th1 cells in the spleen was significantly decreased by 1,25(OH)₂D₃ addition in both VDR-/− and WT mice. However, the proportion of Th1 cells was restored by VDR knockout with addition of 1,25(OH)₂D₃. In contrast, 1,25(OH)₂D₃ addition increased the proportion of Th2 cells in the spleen in both
VDR-/- and WT mice. VDR knockout decreased the proportion of Th1 cells with addition of 1,25(OH)2D3. These results indicated that the effect of 1,25(OH)2D3 on Th1 cell differentiation and cytokine production in vitro and in vivo was mediated by VDR.

Naive CD4+ T cells comprise a heterogeneous cell population, mediate inflammatory responses, and protect against a wide range of pathogens by adopting a series of distinct differentiated states. CD4+ T cells can differentiate into one of two effector cell subsets, Th1 and Th2. Th1 cells mainly secrete IFN-γ, IL-2 and TNF-β and are primarily involved in the inflammatory response of infiltrating mononuclear cells together with specific cell toxicity reaction [20]. Our results showed that both the differentiation of Th1 cells and production of relative cell cytokines (IL-2, IFN-γ and TNF-β) were inhibited by 1,25(OH)2D3. The addition of 1,25(OH)2D3 has direct effects on CD4+ T cells and supports its potent immunosuppressive benefits in the treatment of a number of other autoimmune diseases. Our results are consistent with previous research reporting that 1,25(OH)2D3 had direct effects on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol 2001; 167: 4974-4980.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

VD, Vitamin D; VDR, Vitamin D receptor; BCG, Bacillus Calmette Guerin; IL-2, Interleukin-2; IFN-γ, Interferon-γ; TNF-β, Tumor Necrosis Factor β; JAK, Janus Kinase; Th1, The helper cell type1.

Address correspondence to: Drs. Dong Sun and Jianzhong Xu, Department of Orthopedics, Southwest Hospital, Third Military Medical University, Chongqing, China. E-mail: sumersun07@126.com (DS); xjzslw@163.edu (JZX)

References

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