Effect of 1,25-Dihydroxyvitamin D3 on Th17 and Th1 Response in Patients with Behçet's Disease

Yuan Tian,[1,2] *Chaokui Wang,*[1,2] *Zi Ye,*[1] *Xiang Xiao,*[1] *Aize Kijlstra,*[3] *and Peizeng Yang*[1]

Purpose. 1,25-Dihydroxyvitamin D3 (VitD3) has been shown to have immunoregulatory properties in animal models. In this study, we investigated its inhibitory effect on the immune response in Behçet's disease (BD) patients and the possible mechanisms involved.

Methods. Naive $CD4^+$ T cells from active BD patients and normal controls were cultured under Th17 polarizing conditions in the presence or absence of VitD3, and cytokine production was determined by ELISA and flow cytometry. mRNA expression of several factors related to Th17 cell function was determined by real-time PCR. RNA interference for IFN regulatory factor 8 (IRF-8) was performed to study whether it was involved in the inhibitory effect of VitD3 on Th17 cell differentiation. The effect of VitD3-treated dendritic cells (DCs) on $CD4^+$ T cell response was determined by ELISA and flow cytometry.

Results. Stimulation of naive $CD4^+$ T cells under Th17 polarizing conditions showed a higher Th17 cell differentiation in active BD patients. The addition of VitD3 significantly inhibited Th17 cell differentiation both in BD patients and in normal controls. The knockdown of IRF-8 by RNA interference significantly decreased the suppressive effect of VitD3 on Th17 differentiation. VitD3 was able to inhibit the gene expression of RORC, IL-17, IL-23R, CCR6, and Th1 cell differentiation, but upregulated IL-10 expression. VitD3-treated DCs significantly inhibited the Th17 and Th1 response.

Conclusions. The findings suggest that the inhibitory effect of VitD3 on the Th17 and Th1 response was mediated via both T cells and DCs and that the IRF-8 pathway is involved in the direct inhibition of VitD3 on Th17 cell differentiation. (*Invest Ophthalmol Vis Sci.* 2012;53:6434–6441) DOI:10.1167/iovs.12-10398

From [1]The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, Chongqing Eye Institute, Chongqing, People's Republic of China; and the [3]Eye Research Institute Maastricht, Department of Ophthalmology, University Hospital Maastricht, Maastricht, The Netherlands.

[2]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by Key Project of the Natural Science Foundation (81130019), National Basic Research Program of China (973 Program) (2011CB510200), National Natural Science Foundation Project (30973242), Chongqing Key Laboratory of Ophthalmology (CSTC, 2008CA5003), Program for the Training of a Hundred Outstanding S&T Leaders of Chongqing Municipality, and Fund for PAR-EU Scholars Program. The authors alone are responsible for the content and writing of the paper.

Submitted for publication June 14, 2012; revised August 1 and 12, 2012; accepted August 20, 2012.

Disclosure: **Y. Tian**, None; **C. Wang**, None; **Z. Ye**, None; **X. Xiao**, None; **A. Kijlstra**, None; **P. Yang**, None

Corresponding author: Peizeng Yang, The First Affiliated Hospital of Chongqing Medical University, Youyi Road 1, Chongqing, 400016, People's Republic of China; peizengycmu@126.com.

1a,25-Dihydroxyvitamin D3 (1a,25-(OH)2D3; VitD3) is a secosteroid hormone that binds to a nuclear receptor named vitamin D3 receptor (VDR). VDR is expressed on antigen presentation cells (APCs) such as macrophages and dendritic cells (DC), as well as on both $CD4^+$ T and $CD8^+$ T lymphocytes.[1,2] It is well known that VitD3 controls bone and calcium metabolism. Recent studies using experimental autoimmune models have shown that VitD3 analogs possess immunoregulatory properties. A VitD3 analog was shown to prevent and to partially reverse inflammation in the experimental autoimmune uveitis (EAU, a mouse model for human uveitis) model through its inhibitory effects on the Th17 cell response.[3] In collagen-induced arthritis (CIA, a mouse model for human rheumatoid arthritis), VitD3 and VitD3 analogs prevented the development of arthritis and diminished CIA severity.[4] In experimental autoimmune encephalomyelitis (EAE, model for human multiple sclerosis), VitD3 significantly reduced EAE severity by inhibiting chemokine synthesis and monocyte trafficking.[5]

A number of studies have demonstrated that VitD3 could modulate the activity of DCs. It was found that VitD3 inhibited the differentiation and maturation of DCs; reduced the expression of major histocompatibility complex (MHC) class II, CD40, CD80, and CD86; and inhibited the secretion of proinflammatory cytokines, including IL-1, IL-6, TNF-α, and IL-12.[6] In addition, VitD3 induced the development of IL-10-producing $CD4^+$ T cells[7] and stimulated the Th2 polarization from naive $CD4^+$ T cells.[8] A study by our lab reported that VitD3 was decreased in active uveitis patients with Vogt-Koyanagi-Harada (VKH) disease and that VitD3 was able to inhibit the production of IL-17 and IFN-γ by peripheral blood mononuclear cells (PBMC) and $CD4^+$ T cells.[9] Recently, VitD3 analogs have been used as topical treatment for psoriasis,[10] and clinical trials for its use in autoimmune diseases are under way.[11,12] However, the mechanisms involved in the inhibition of VitD3 on the human immune response are not well understood. A study was therefore designed to investigate the influence of VitD3 on differentiation of Th17 and Th1 cells, production of molecules related to Th17 differentiation and function, DC function, and the possible mechanisms involved in these processes in Behçet's disease (BD), a well-known autoinflammatory disease mediated by both Th17 and Th1 cells.[13]

In this study, we found that VitD3 inhibited differentiation of Th17 and Th1 cells from naive $CD4^+$ T cells under Th17 polarizing conditions both in BD patients and in normal controls. Interestingly, we found that IFN regulatory factor 8 (IRF-8) played a key role in the direct inhibitory effect of VitD3 on Th17 differentiation. We also found that VitD3 inhibited the expression of Th17 cell effector molecules such as *RORC*, *IL-17*, *IL-23R*, and *CCR6*. On the other hand, VitD3 stimulated the secretion of regulatory IL-10 by naive $CD4^+$ T cells. Finally, we showed that DCs were involved in the VitD3-mediated suppression of the Th1 and Th17 cell response.

Copyright 2012 The Association for Research in Vision and Ophthalmology, Inc.

Downloaded from iovs.arvojournals.org on 05/17/2022

Materials and Methods

Subjects

Fifteen active BD patients (8 men and 7 women, with an average age of 37.4 years) and 18 normal controls (10 men and 8 women, with an average age of 35 years) were included in this study. Patients were diagnosed as having BD according to the diagnostic criteria designed by the International Study Group for Behçet's Disease.[14] Patients showed active intraocular inflammation as evidenced by nongranulomatous keratic precipitates (100%), flare and cells in the anterior chamber (100%), vitreous cells (81%), and retinal vasculitis (100%) disclosed by fundus fluorescein angiography (FFA). The extraocular manifestations were recurrent oral aphthous ulcers (100%), multiform skin lesions (85%), recurrent genital ulcers (37.5%), and arthritis (37.5%). No immunosuppressive agents or prednisone was given to these patients before their visits to us and blood sampling. Written and informed consent was obtained from all patients and normal controls. All procedures followed the tenets of the Declaration of Helsinki and were approved by the Clinical Ethical Research Committee of Chongqing Medical University.

Cell Culture

PBMCs were prepared from heparinized blood by Ficoll-Hypaque density-gradient centrifugation. Naive $CD4^+$ T cells were separated from PBMCs by magnetic microbead negative selection according to the manufacturer's instructions (the purity of $CD4^+CD45RA^+$ T cells was 93%; Miltenyi Biotec, Palo Alto, CA). To study the effect of VitD3 on Th17 polarization, naive $CD4^+$ T cells were stimulated at 1×10^6/mL with anti-CD3 and anti-CD28 (2 ug/mL) (eBioscience, San Diego, CA) in Th17 polarizing conditions (IL-6, 50 ng/mL; IL-23, 10 ng/mL; IL-1β, 10 ng/mL; anti IL-4, 10 ug/mL; and anti–IFN-γ, 10 ug/mL) in the presence or absence of VitD3 (10 nM) (Sigma-Aldrich, St. Louis, MO). On day 3, rIL-2 (100 ng/mL) was added. The VitD3 was dissolved in ethanol at an initial 1 mM concentration and serially diluted to the working concentrations with RPMI 1640 medium. All antibodies (Abs) and recombinant cytokines used in the Th17 cell polarization experiments were purchased from R&D Systems (Minneapolis, MN). On day 7, the supernatants were obtained and the concentrations of IL-17, IFN-γ, and IL-10 were measured by ELISA (R&D Systems). The cells were harvested for intracellular detection of cytokines by flow cytometry or for gene expression assays.

$CD14^+$ monocytes and $CD4^+$ T cells were separated from PBMCs using human microbeads (both purity >90%; Miltenyi Biotec). DCs were generated from monocytes plated at 1×10^6 cells/mL in RPMI 1640 complete medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), and penicillin G/streptomycin (100 U/mL; Invitrogen, Carlsbad, CA) in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/mL; R&D Systems) and IL-4 (50 ng/mL; R&D Systems). VitD3 was added at the beginning of the culture at a concentration of 10 nM. On day 3, half of the culture medium including recombinant cytokines and VitD3 was refreshed. On day 6, the cells were stimulated with lipopolysaccharide (LPS) for 24 hours, and matured DCs were washed three times before coculturing with $CD4^+$ T cells. Cocultures were performed with a DC:T cell ratio of 1:5 in 96-well plates for 4 days. The culture supernatants were assayed for IL-17 and IFN-γ levels by ELISA. The cells were harvested for intracellular detection of IL-17 and IFN-γ using flow cytometry.

RNA Interference

Naive $CD4^+$ T cells were transfected with 100 nM small interfering RNA (siRNA) specific for IRF-8 and a negative control (Qiagen, Valencia, CA) using human T cell Nucleofector kit (Amaxa; Lonza, Basel, Switzerland). After transfection, the cells were incubated for 4 hours at 37°C and then stimulated with anti-CD3 and anti-CD28 under Th17 polarizing conditions in the presence or absence of VitD3 (10 nM) for 48 hours. Cells were harvested at 48 hours to detect the expression of IRF-8 mRNA. Cells were harvested for intracellular detection of IL-17 by flow cytometry, and IL-17 in the supernatants was measured by ELISA.

Real-Time Quantitative RT-PCR

Total RNA was extracted from stimulated naive $CD4^+$ T cells using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The first-strand cDNA was synthesized for each RNA sample using the Superscript III Reverse Transcriptase system (Invitrogen). Real-time quantitative PCR was performed on AB 7500 Fast System (Applied Biosystems, Foster City, CA). The following primers were used for real-time PCR: IL-23R forward 5′-AACAGCAATGTTGTGAAAATGC3′, reverse 5′-AGGCTTGTGTTCTGGGATGA-3′; IL-17 forward 5′-ACCAATCCCAAAAGGTCCTC-3′, reverse 5′-TGGATGGGGACAGAGTTCAT-3′; RORC2 forward 5′- CAGTCATGAGAACACAAATTGAAGTG-3′, reverse 5′-CAGGTGATAACCCCGTAGTGGAT-3′; CCR6 forward 5′-GGCTGCAAATTTGGGTAAAA-3′, reverse 5′-CACAGGAGAAGCCTGAGGAC-3′; IRF-8 forward 5′-GAAGACGAGGGTTACGCTGTG-3′, reverse 5′-TCCTCAGGAACAATTCGGTAA-3′; β-actin forward 5′-GGATGCAGAAGGAGATCACTG-3′, reverse 5′-CGATCCACACGGAGTACTTG-3′. The expression of each gene was normalized to the expression of β-actin using the $2^{-\Delta\Delta CT}$ method as described previously.[15]

Enzyme-Linked Immunoabsorbent Assay

Supernatants from naive $CD4^+$ T cells and DC/T cell cocultures were collected and stored at −80°C for future cytokine measurements. IL-17, IFN-γ, and IL-10 secretion were measured with ELISA kits from R&D Systems according to the manufacturer's instructions.

Flow Cytometric Analysis

For analysis of intracellular cytokine levels in naive $CD4^+$ T cells and $CD4^+$ T cells cocultured with VitD3-treated DCs or control DCs by flow cytometry, we used the following fluorochrome-conjugated Abs: anti-human CD3-(PerCP)-Cy5.5, anti-human CD8-APC, anti-human IL-17A-PE, anti-human IFN-γ-FITC, and mouse anti-human IgG isotype control Ab. All fluorescent Abs were obtained from BD Biosciences (Sunnyvale, CA). The cells were stimulated by addition of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (1 ug/mL; Sigma) for 1 hour at 37°C. Then, brefeldin A (10 ug/mL; Sigma) was added for another 4 hours. The cells were fixed and permeabilized using the eBioscience Cytofix/Cytoperm kit according to the manufacturer's instructions and then incubated with the above fluorescent Abs. The cells were washed and analyzed on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Statistical Analysis

Student's *t*-test and ANOVA were applied using SPSS 10.0 software (SPSS Inc., Chicago, IL). Data are shown as mean ± SD. *P* values less than 0.05 were considered statistically significant.

Results

VitD3 Inhibits IL-17 Production and Th17 Cell Differentiation in Both BD Patients and Normal Controls

Previous studies have revealed that VitD3 has a suppressive effect on Th17 cell differentiation in animals.[3] In this study, we examined the influence of VitD3 on Th17 cell differentiation and the production of IL-17 by polarized naive $CD4^+$ T cells in

Downloaded from iovs.arvojournals.org on 05/17/2022

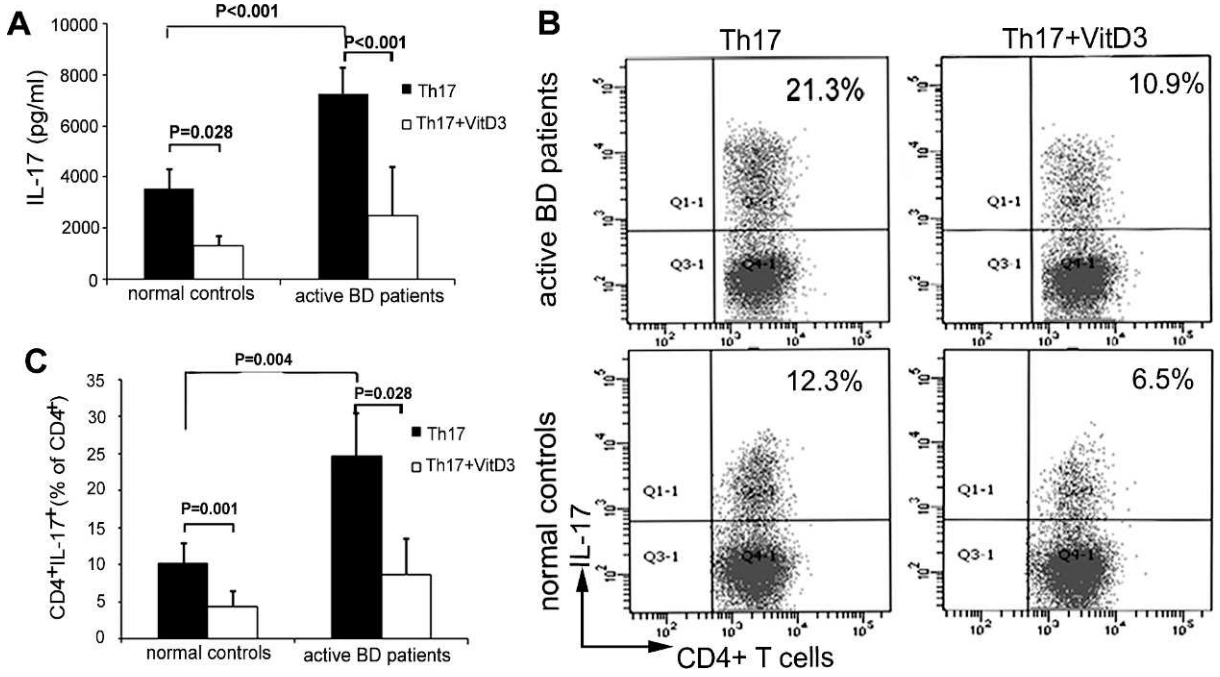

Figure 1. VitD3 inhibits Th17 cell differentiation in BD patients and normal controls. Naive $CD4^+$ T cells from active BD patients ($n = 6$) and normal controls ($n = 6$) were cultured under Th17 polarizing conditions with or without VitD3 (10 nM) for 6 days. (**A**) ELISA of IL-17 production in the supernatants. Flow cytometric analysis of IL-17 on the stimulated naive $CD4^+$ T cells. (**B**) Representative dot plots and (**C**) quantitative analysis of the frequency of IL-17-producing $CD4^+$ T cells after PMA/ionomycin stimulation. Data are expressed as mean ± SD.

both BD patients and normal controls. Highly purified naive $CD4^+$ T cells were cultured under Th17 polarizing conditions in the presence or absence of VitD3. The results showed that IL-17 production in the supernatants of stimulated naive $CD4^+$ T cells was significantly higher in BD patients than in normal controls ($P < 0.001$). Addition of VitD3 significantly inhibited the production of IL-17 in both BD patients and normal controls ($P < 0.001$ and $P = 0.028$, respectively) (Fig. 1A).

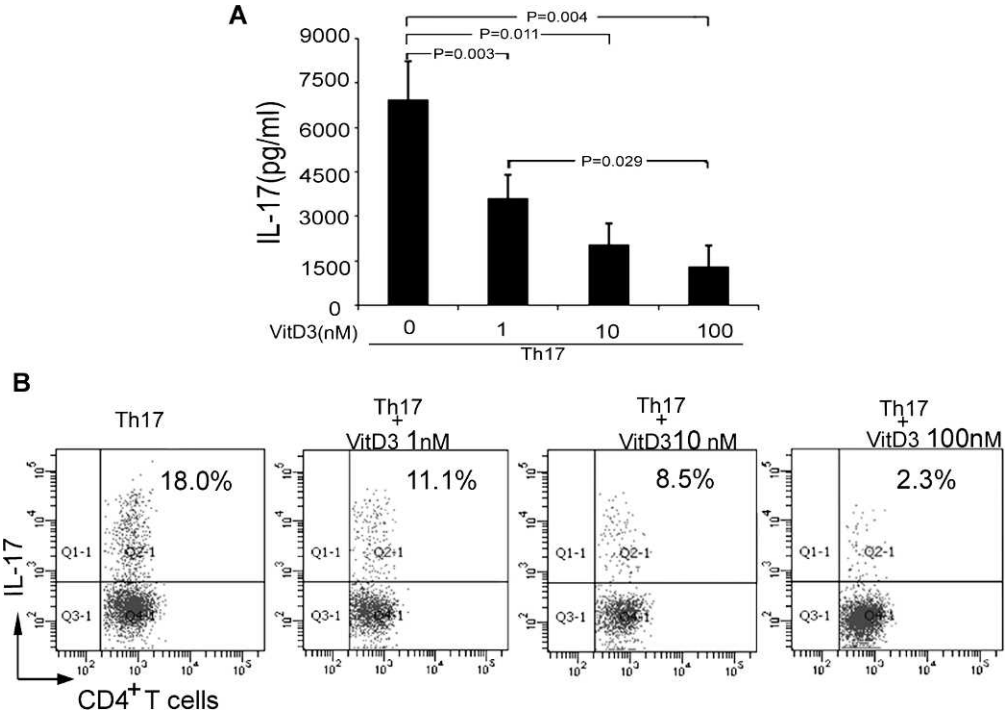

Figure 2. VitD3 inhibits Th17 cell differentiation in a dose-dependent manner. Naive $CD4^+$ T cells from BD patients ($n = 5$) were cultured under Th17 polarizing conditions with different concentrations of VitD3 (0, 1, 10, 100 nM) for 6 days. (**A**) ELISA of the IL-17 production in the supernatants. Flow cytometric analysis of IL-17 on the stimulated naive $CD4^+$ T cells, (**B**) dot plots of a representative subject. Data are expressed as mean ± SD.

Downloaded from iovs.arvojournals.org on 05/17/2022

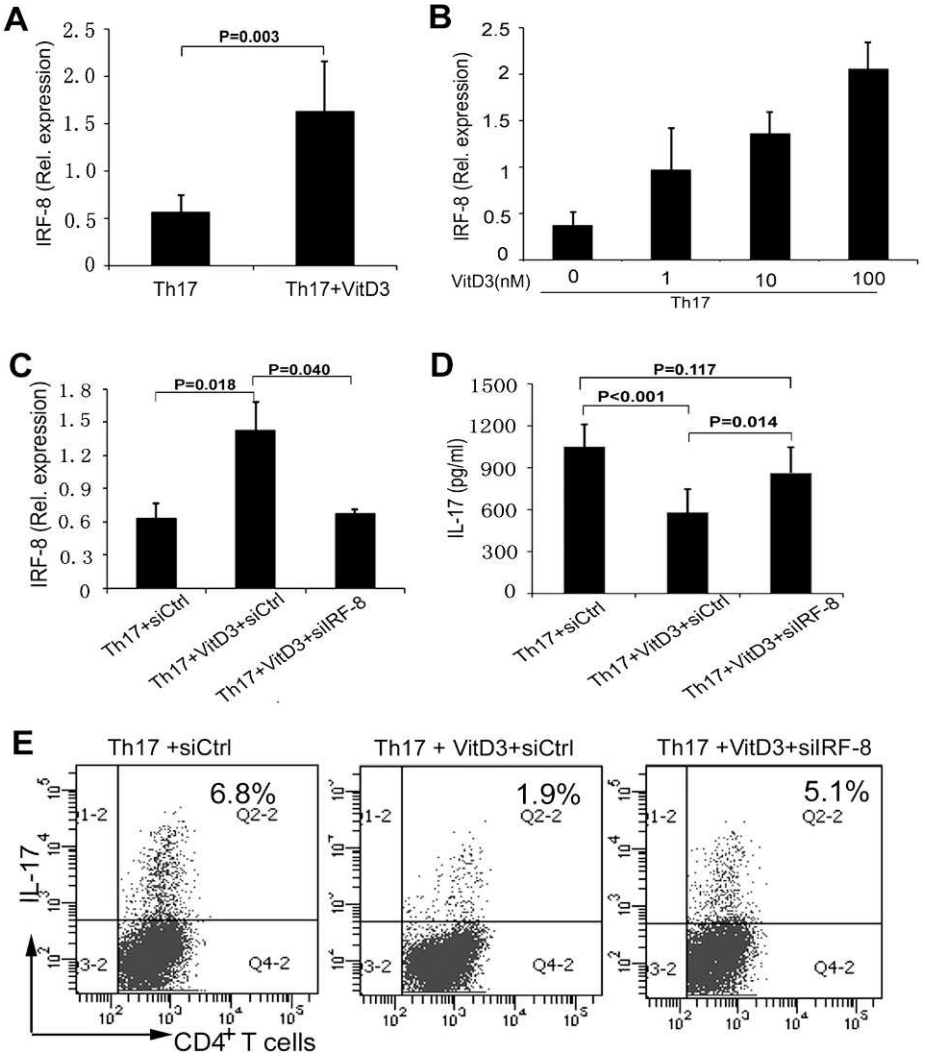

Figure 3. VitD3 inhibits Th17 cell differentiation by upregulating IRF-8. Real-time RT-PCR analysis of IRF-8 expression in naive $CD4^+$ T cells from normal controls ($n = 5$) cultured in Th17 polarizing conditions (**A**) with or without VitD3 (10 nM) or (**B**) with different concentrations of VitD3 (0, 1, 10, 100 nM). (**C**) Real-time PCR analysis of IRF-8 expression in the naive $CD4^+$ T cells from normal controls ($n = 6$) nucleoporated with IRF-8-specific siRNA or control siRNA and then cultured under Th17 polarizing conditions in the presence or absence of VitD3 (10 nM). (**D**) ELISA of IL-17 in the supernatants of these cultured naive $CD4^+$ T cells. (**E**) Dot plots of a representative subject. Data are expressed as mean ± SD.

Consistent with ELISA data, intracellular cytokine analysis revealed that a significantly increased frequency of IL-17-producing $CD4^+$ T cells was observed in BD patients as compared with normal controls ($P = 0.004$). VitD3 could also significantly inhibit the frequency of IL-17-producing $CD4^+$ T cells in BD patients and normal controls ($P = 0.028$ and $P = 0.001$, respectively) (Figs. 1B, 1C). Our results also showed that the inhibition of VitD3 on IL-17 production and the frequency of IL-17-producing $CD4^+$ T cells was dose dependent (Fig. 2).

VitD3 Inhibits Th17 Cell Differentiation through Upregulating IRF-8

The aforementioned results showed that VitD3 inhibited Th17 cell differentiation. A recent study reported that transcriptional factor IRF-8 was expressed in Th17 cells and that it plays a negative role in Th17 cell differentiation.[16] In view of these findings, we examined whether VitD3 exerted its inhibitory effect on Th17 cell differentiation by modulating IRF-8. We stimulated naive $CD4^+$ T cells under Th17 polarizing conditions in the presence or absence of VitD3. The results showed that VitD3 significantly induced the gene expression of IRF-8 ($P = 0.003$, Fig. 3A). Furthermore, the observed stimulatory effect was dose dependent (Fig. 3B). Using the method of RNA interference, we found that the inhibitory effect of VitD3 on the production of IL-17 by stimulated naive $CD4^+$ T cells was significantly abrogated when IRF-8 was silenced (Figs. 3C, 3D). Similar to the ELISA, intracellular cytokine analysis revealed that the inhibitory effect of VitD3 on the frequency of polarized IL-17-producing $CD4^+$ T cells was also decreased when IRF-8 was silenced in naive $CD4^+$ T cells (Figs. 3C, 3E).

VitD3 Inhibits the Molecules Associated with Th17 Cell Differentiation or Effector Function in Naive $CD4^+$ T cells

It has been demonstrated that the nuclear receptor RORC is a key transcription factor for human Th17 cell lineage commitment[17] and that IL-23R and CCR6 are essential to the

Downloaded from iovs.arvojournals.org on 05/17/2022

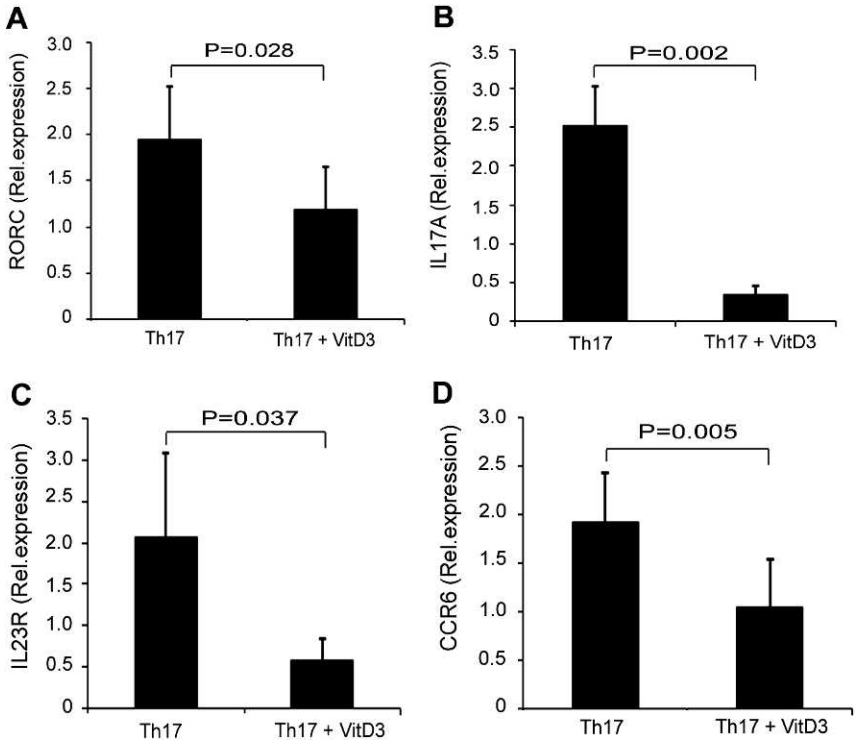

FIGURE 4. VitD3 inhibits the molecules associated with Th17 cell effector function. Naive $CD4^+$ T cells from BD patients ($n = 5$) were cultured under Th17 polarizing conditions with VitD3 (10 nM) or without VitD3 for 6 days. The cells were harvested for mRNA analysis for (**A**) RORC expression, (**B**) IL-17 expression, (**C**) IL-23R expression, and (**D**) CCR6 expression by real-time PCR.

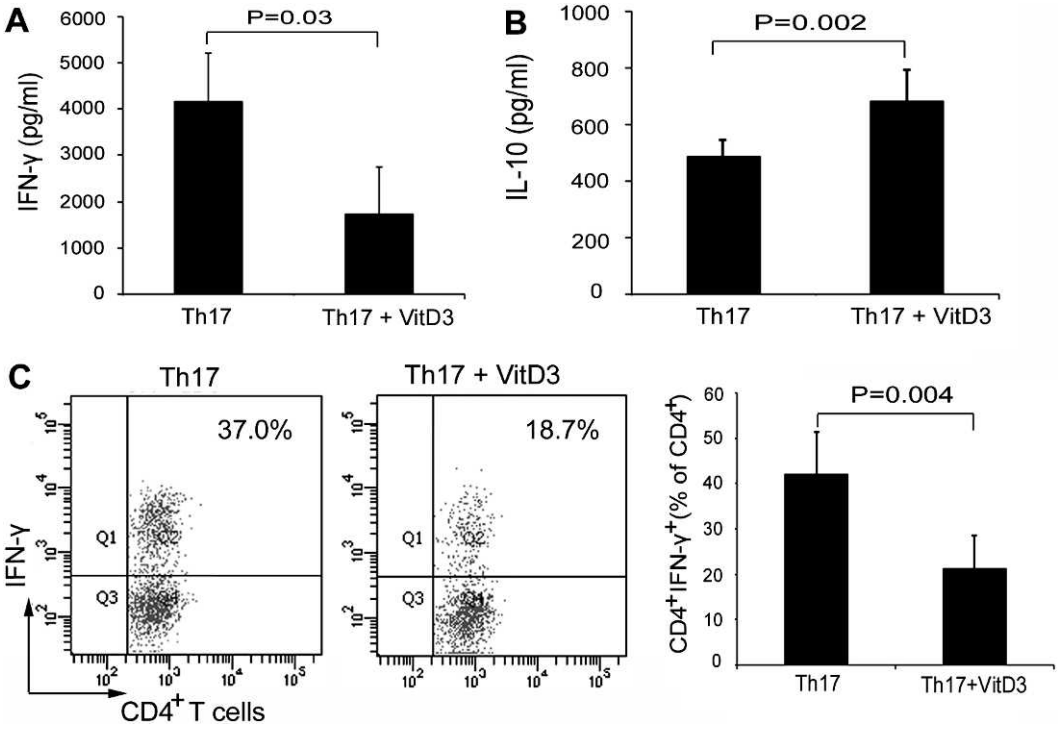

FIGURE 5. VitD3 downregulates the expression of IFN-γ, but promotes secretion of IL-10 by naive $CD4^+$ T cells cultured in Th17 polarizing conditions. (**A**, **B**) ELISA of IFN-γ and IL-10 in the supernatants of cultured naive $CD4^+$ T cells with or without VitD3 (10 nM) derived from BD patients ($n = 5$). (**C**) Representative dot plots and (**D**) quantitative analysis of the frequency of IL-17-producing $CD4^+$ T cells after PMA/ionomycin stimulation. Data are expressed as mean ± SD.

Downloaded from iovs.arvojournals.org on 05/17/2022

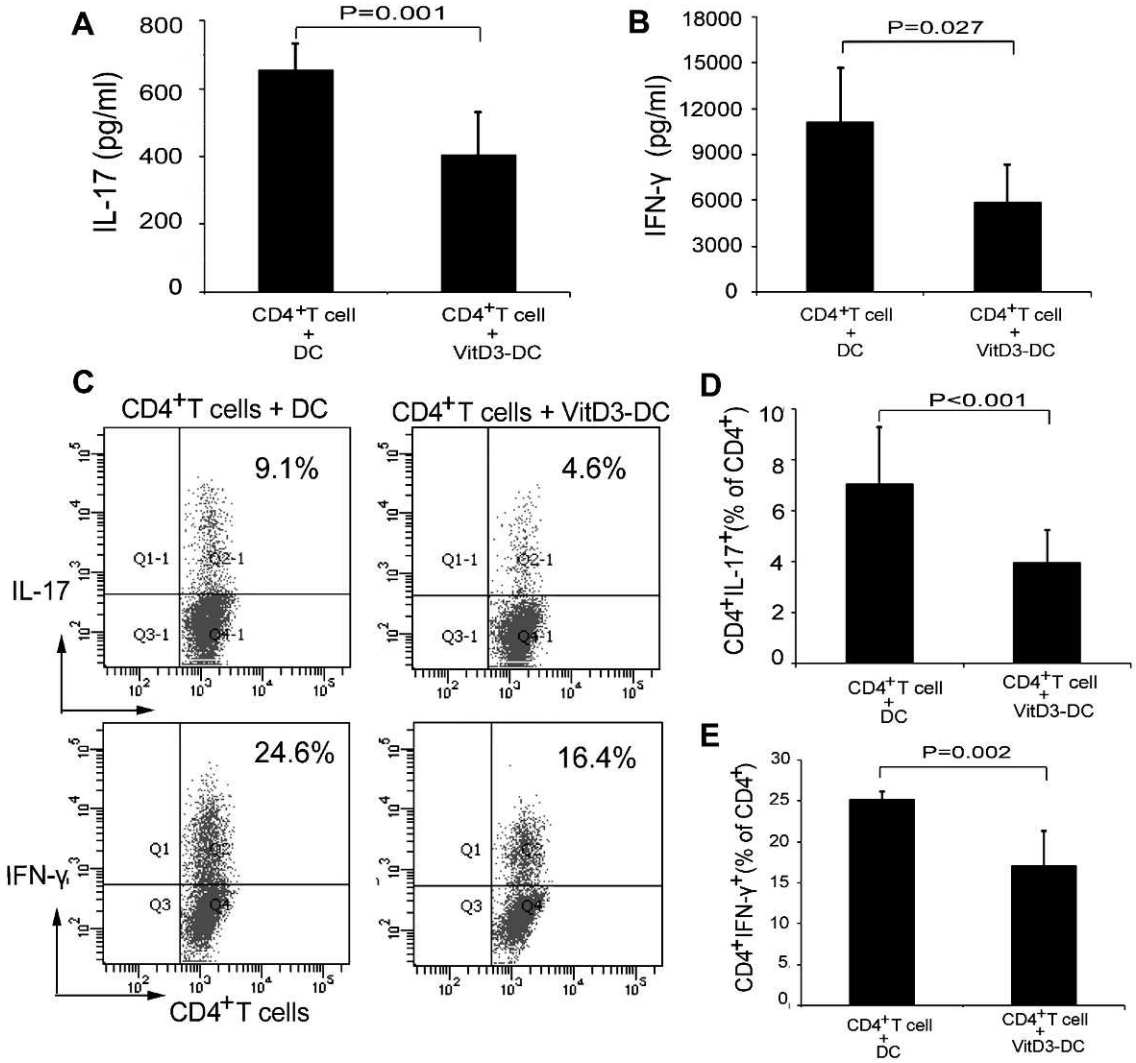

Figure 6. VitD3-treated DCs have a decreased ability to induce Th1 and Th17 cell response. Allogeneic $CD4^+$ T cells were isolated from BD patients ($n = 6$) and cocultured with control DCs and VitD3-treated DCs for 7 days (VitD3 10 nM). (**A**, **B**) ELISA of IL-17 and IFN-γ in the supernatants. (**C**) Representative dot plots and (**D**, **E**) quantitative analysis of the frequency of IL-17- and IFN-γ-producing $CD4^+$ T cells after PMA/ionomycin stimulation. Data are expressed as mean ± SD.

maintenance and function of Th17 cells.[18,19] In this study, we examined the effect of VitD3 on these molecules in naive $CD4^+$ T cells under Th17 polarizing conditions. The results showed that VitD3 significantly inhibited the gene expression of the molecules related to differentiation of Th17 cells *RORC* ($P = 0.028$, Fig. 4A) and *IL-17* ($P = 0.002$, Fig. 4B). Similarly, it was also able to inhibit the gene expression of molecules related to the function of Th17 cells such as *IL-23R* ($P = 0.037$, Fig. 4C) and *CCR6* ($P = 0.005$, Fig. 4D).

VitD3 Inhibits the Expression of IFN-γ, but Promotes Secretion of IL-10 by Naive $CD4^+$ T Cells

We further investigated the effect of VitD3 on the expression of IFN-γ and IL-10 by naive $CD4^+$ T cells under Th17 polarizing conditions. The results showed that VitD3 significantly inhibited the production of IFN-γ ($P = 0.03$, Fig. 5A), but induced a significant increase in the production of IL-10 ($P = 0.002$, Fig. 5B). Using intracellular cytokine staining, we observed that the frequency of IFN-γ-producing T cells was also inhibited when naive $CD4^+$ T cells were cultured under Th17 polarizing conditions in the presence of VitD3 ($P = 0.004$, Fig. 5C). Moreover, the frequency of IFN-γ and IL-17 double-positive $CD4^+$ T cells was also inhibited when naive $CD4^+$ T cells were cultured under Th17 polarizing conditions in the presence of VitD3 (data not shown).

VitD3-Treated DCs Have a Decreased Ability to Induce Th1 and Th17 Cell Response

To investigate the effect of VitD3-treated DCs on the development of $CD4^+$ T cells, we cocultured $CD4^+$ T cells with control DCs and VitD3-treated DCs and determined the levels of IL-17 and IFN-γ in the cell supernatants by ELISA. The results showed that the expression of IL-17 and IFN-γ was significantly decreased in the supernatants of naive $CD4^+$ T cells cocultured with VitD3-treated DCs as compared with those cocultured with control DCs ($P = 0.001$ and $P = 0.027$, respectively; Figs. 6A, 6B). Flow cytometry showed that the frequency of IL-17- and IFN-γ-staining $CD4^+$ T cells was also decreased in the naive $CD4^+$ T cells cocultured with VitD3-treated DCs as compared with those cultured with control DCs ($P < 0.001$ and $P = 0.002$, respectively; Figs. 6C–E).

Downloaded from iovs.arvojournals.org on 05/17/2022

Discussion

In this study, we showed that VitD3 inhibited Th1 and Th17 cell differentiation in BD patients. We provided evidence that VitD3 inhibited Th17 differentiation via an IRF-8 pathway. We also found that VitD3 inhibited various molecules associated with the differentiation and function of Th17 cells such as *RORC*, *CCR6*, and *IL-23R*. VitD3 also inhibited Th1 differentiation, but induced IL-10 production by naive $CD4^+$ T cells under Th17 polarizing conditions. Our in vitro data suggest that VitD3 inhibits Th1 and Th17 cell response through DCs. These results suggest that the use of VitD3 to control these responses may lead to an addition to the therapeutic repertoire to treat this blinding disease.

As Th17 and Th1 cells are essential to the development of autoimmune or autoinflammatory disease,[20–26] it is important to unravel the various pathways employed by these cells, allowing the development of various therapeutic approaches that could potentially be used for treatment of these diseases. VitD3 or its analogs have already been shown to suppress the Th17 effector response in animals.[3] Boonstra et al. recently found that VitD3 could inhibit Th1 polarization but enhanced Th2 cell development (IL-10, IL-4, and IL-5 production).[8] Furthermore, a recent study by our lab showed that VitD3 was able to inhibit IL-17 and IFN-γ production by stimulated PBMCs in patients with Vogt-Koyanagi-Harada disease.[9] These results collectively suggest that VitD3 inhibits both the Th17- and the Th1-mediated immune response in humans. In this investigation, we first studied the effect of VitD3 on the differentiation of Th17 cells. The results showed that VitD3 could inhibit the frequency of IL-17-producing $CD4^+$ T cells and IL-17 secretion by naive $CD4^+$ T cells under Th17 polarizing conditions. These findings validate the suppressive effect of VitD3 on IL-17 secretion. Moreover, naive $CD4^+$ T cells from active BD patients were more sensitive to VitD3 as compared to normal controls. These results seem to suggest that there was a higher level of VitD3 receptor or receptor affinity in the stimulated naive $CD4^+$ T cells from active BD patients. The concentration of VitD3 used in our in vitro study was 10 nM (4.1664 ng/mL). A recent study by Hamzaoui et al. found that the level of VitD3 in normal controls was 14.03 ± 5.20 ng/ml.[27] Therefore, the concentration of VitD3 used in our study is relevant to the physiological level of VitD3.

The exact mechanism whereby VitD3 affects Th17 cell differentiation is not well known. It has been reported that the transcriptional factor IRF-8 is expressed in Th17 cells and is involved in Th17 cell differentiation.[16] We therefore investigated whether VitD3 exerted its role through modulating the expression of IRF-8. The results showed that VitD3 could significantly induce the gene expression of IRF-8. Additionally, we found that specific inhibition of IRF-8 gene expression using IRF-8 siRNA abrogated the effect of VitD3-mediated inhibition of Th17 differentiation. Previous studies reported that several mechanisms may be involved in the VitD3-mediated inhibition on Th17 cells. C/EBP homologous protein (CHOP), a molecule involved in endoplasmic reticulum stress and translational inhibition, has been shown to be involved in the inhibitory effect of VitD3 on Th17 cytokine production.[28] Furthermore, nuclear factor for activated T cells (NFAT), histone deacetylase (HDAC), and Runt-related transcription factor 1 (Runx1) were all involved in the controlling effect of 1,25(OH)(2)D(3) on IL-17A production.[29] Our results add a further regulatory mechanism involving the IRF-8 pathway.

As numerous molecules, such as RORC, CCR6, and IL-23R, are related to the differentiation and function of Th17 cells, it is interesting to investigate the influence of VitD3 on these factors. The nuclear receptor RORC is a key transcription factor in the Th17 lineage commitment process.[17] We found that the addition of VitD3 to naive $CD4^+$ T cells under Th17 polarizing conditions resulted in a significant decrease in the gene expression of RORC and IL-17. Our results are in disagreement with earlier animal studies, which showed that VitD3 inhibited the gene expression of IL-17 without influencing the gene expression of RORγt.[3] The discrepant findings may be due the different cell types used in these studies or due to species differences. Previous animal studies have shown that IL-23 is required for Th17 cells to keep a stable population and maintain a pathogenic function.[30,31] Human Th17 cells have been described as having a constitutive expression of IL-23R on their cell surface.[18] We found that VitD3 markedly inhibited the gene expression of IL-23R, suggesting that VitD3 can inhibit the expansion and pathogenicity of the Th17 cells. CCR6 is expressed on human Th17 cells and contributes to their recruitment to sites of inflammation.[19,32] We found that the addition of VitD3 to naive $CD4^+$ T cells cultured under Th17 polarizing conditions inhibited the gene expression of CCR6, suggesting that VitD3 can decrease the number of pathogenic Th17 cells at the site of inflammation by influencing the migration of Th17 cells to the site. Therefore, the results showed that VitD3 not only inhibited the Th17 cell differentiation, but also inhibited the molecules that are relevant to Th17 cell function and migration.

The aforementioned results showed that VitD3 directly inhibited Th17 and Th1 cell differentiation. It is not yet known whether VitD3 could exert its effect on Th17 and Th1 cells in humans via an interaction with DCs. DCs are critical to the differentiation of Th17 and Th1 cells and their function.[33–35] A further study was therefore designed to examine whether VitD3 has an indirect effect on the development of Th17 and Th1 cells through DCs. The results showed that VitD3-treated DCs could significantly inhibit Th17 and Th1 cell response from $CD4^+$ T cells when they were cocultured. Our results are in agreement with earlier findings in animals showing that VitD3 was able to inhibit Th17 cell differentiation through DCs in vivo.[3] Based on our studies and previous ones, it becomes evident that VitD3 acts as a negative modulator of both Th17 and Th1 cell response via an effect on DCs.

Our findings together with animal studies suggest that VitD3 can potentially modulate an aberrant immune response and may therefore present a novel therapeutic approach for BD and other autoimmune or autoinflammatory diseases mediated by an abnormal Th17 and Th1 immune response.

References

1. Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J*. 2001;15:2579–2585.
2. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1, 25-dihydroxyvitamin D(3) receptor in the immune system. *Arch Biochem Biophys*. 2000;374:334–338.
3. Tang J, Zhou R, Luger D, et al. Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. *J Immunol*. 2009;182:4624–4632.
4. Cantorna MT, Hayes CE, DeLuca HF. 1, 25-Dihydroxycholecalciferol inhibits the progression of arthritis in murine models of human arthritis. *J Nutr*. 1998;128:68–72.
5. Pedersen LB, Nashold FE, Spach KM, Hayes CE. 1, 25-dihydroxyvitamin D3 reverses experimental autoimmune encephalomyelitis by inhibiting chemokine synthesis and monocyte trafficking. *J Neurosci Res*. 2007;85:2480–2490.
6. Piemonti L, Monti P, Sironi M, et al. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol*. 2000;164:4443–4451.
7. Fujio K, Okamura T, Yamamoto K. The family of IL-10-secreting CD4+ T cells. *Adv Immunol*. 2010;105:99–130.

Downloaded from iovs.arvojournals.org on 05/17/2022

8. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha, 25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol*. 2001;167:4974–4980.
9. Yi X, Yang P, Sun M, Yang Y, Li F. Decreased 1, 25-Dihydroxyvitamin D3 level is involved in the pathogenesis of Vogt-Koyanagi-Harada (VKH) disease. *Mol Vis*. 2011;17:673–679.
10. van de Kerkhof PC, Wasel N, Kragballe K, Cambazard F, Murray S. A two-compound product containing calcipotriol and betamethasone dipropionate provides rapid, effective treatment of psoriasis vulgaris regardless of baseline disease severity. *Dermatology*. 2005;210:294–299.
11. Franceschini N, Joy MS, Kshirsagar A. Cinacalcet HCl: a calcimimetic agent for the management of primary and secondary hyperparathyroidism. *Expert Opin Investig Drugs*. 2003;12:1413–1421.
12. Arnson Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. *Ann Rheum Dis*. 2007;66:1137–1142.
13. Chi W, Zhu X, Yang P, et al. Upregulated IL-23 and IL-17 in Behcet patients with active uveitis. *Invest Ophthalmol Vis Sci*. 2008;49:3058–3064.
14. International Study Group for Behçet's Disease. Criteria for diagnosis of Behçet's disease. *Lancet*. 1990;335:1078–1080.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
16. Ouyang X, Zhang R, Yang J, et al. Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation. *Nat Commun*. 2011;2:314.
17. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol*. 2008;9:641–649.
18. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med*. 2007;204:1849–1861.
19. Hirota K, Yoshitomi H, Hashimoto M, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med*. 2007;204:2803–2812.
20. Luger D, Silver PB, Tang J, et al. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J Exp Med*. 2008;205:799–810.
21. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol*. 2009;183:7169–7177.
22. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol*. 2006;177:566–573.
23. O'Connor RA, Prendergast CT, Sabatos CA, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol*. 2008;181:3750–3754.
24. Arican O, Aral M, Sasmaz S, Ciragil P. Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm*. 2005;2005:273–279.
25. Matusevicius D, Kivisakk P, He B, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler*. 1999;5:101–104.
26. Amadi-Obi A, Yu CR, Liu X, et al. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med*. 2007;13:711–718.
27. Hamzaoui K, Ben Dhifallah I, Karray E, Sassi FH, Hamzaoui A. Vitamin D modulates peripheral immunity in patients with Behcet's disease. *Clin Exp Rheumatol*. 2010;28:S50–57.
28. Chang SH, Chung Y, Dong C. Vitamin D suppresses Th17 cytokine production by inducing C/EBP homologous protein (CHOP) expression. *J Biol Chem*. 2010;285:38751–38755.
29. Joshi S, Pantalena LC, Liu XK, et al. 1, 25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. *Mol Cell Biol*. 2011;31:3653–3669.
30. Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 2003;421:744–748.
31. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*. 2005;201:233–240.
32. Wang C, Kang SG, Lee J, Sun Z, Kim CH. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol*. 2009;2:173–183.
33. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245–252.
34. Lanzavecchia A, Sallusto F. The instructive role of dendritic cells on T cell responses: lineages, plasticity and kinetics. *Curr Opin Immunol*. 2001;13:291–298.
35. Sallusto F, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. *Arthritis Res*. 2002;4(suppl 3):S127–S132.

Downloaded from iovs.arvojournals.org on 05/17/2022