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# 1$\alpha$,25-Dihydroxyvitamin D3 Has a Direct Effect on Naive CD4$^+$ T Cells to Enhance the Development of Th2 Cells[1]

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**1$\alpha$,25-Dihydroxyvitamin D3 (vitD3) is an immunoregulatory hormone with beneficial effects on Th1 mediated autoimmune diseases. Although the inhibitory effects of vitD3 on macrophages and dendritic cells are well documented, any direct effects of vitD3 on Th cell development are not clearly defined. Using CD4$^+$Mel14$^+$ T cells derived from mice on a BALB/c and a C57BL/6 genetic background we examined the effect of vitD3 on Th cell development. We demonstrated that vitD3 affects Th cell polarization by inhibiting Th1 (IFN-$\gamma$ production) and augmenting Th2 cell development (IL-4, IL-5, and IL-10 production). These effects were observed in cultures driven with splenic APC and Ag, as well as with anti-CD3 and anti-CD28 alone, indicating that CD4$^+$ cells can also be direct targets for vitD3. The enhanced Th2 development by vitD3 was found in both BALB/c and C57BL/6 mice. An increased expression of the Th2-specific transcription factors GATA-3 and c-*maf* correlated with the increased production of Th2 cytokines after vitD3 treatment. The vitD3-induced effects were largely mediated via IL-4, because neutralization of IL-4 almost completely abrogated the augmented Th2 cell development after vitD3 treatment. These findings suggest that vitD3 acts directly on Th cells and can, in the absence of APC, enhance the development of a Th2 phenotype and augment the expression of the transcription factors c-*maf* and GATA-3. Our findings suggest that the beneficial effects of vitD3 in autoimmune diseases and transplantation operate through prevention of strong Th1 responses via the action on the APC, while simultaneously directly acting on the T cell to enhance Th2 cell development.** ***The Journal of Immunology,*** **2001, 167: 4974–4980.**

The CD4$^+$ Th cells can be divided into Th1 and Th2 cells based on their cytokine profile upon antigenic stimulation (1, 2). The Th1 subset produces IFN-$\gamma$ and lymphotoxin and is important for protection against intracellular pathogens and has also been associated with autoimmune pathologies (3). The Th2 subset produces IL-4, IL-5, and IL-13 and is implicated in eradicating helminth and other extracellular parasites, as well as being involved in allergic manifestations. The development of CD4$^+$ T cells into either Th1 or Th2 cells determines the outcome of an immune response, and is primarily directed by cytokines; Th1 cells develop in response to IL-12, whereas IL-4 induces the development of Th2 cells (4–7). Studies on the molecular mechanisms of Th development have demonstrated the importance of Th1 (T-bet) and Th2 (c-*maf* and GATA-3) specific transcription factors in the regulation of the Th1/Th2 balance. c-*maf* increases the expression of IL-4 (8), whereas GATA-3 can induce the expression of a wide range of Th2-specific cytokines (9, 10). Furthermore, forced expression of GATA-3 can elicit the production of Th2-specific cytokines in developing and committed Th1 cells, and inhibit IFN-$\gamma$ production (11–13). The Th1-specific transcription factor T-bet induces the expression of IFN-$\gamma$, and represses IL-4 and IL-5 production in developing and committed Th2 cells (14).

Beside the dominant role of cytokines in Th development, additional factors have been described which affect the Th1-Th2 balance, e.g., the Ag dose, the strength of TCR signaling, and costimulatory signals (7). Furthermore, a number of nonprotein compounds have been reported to influence Th cell development, such as glucocorticoids, eicosanoids, and vitamins (15–19).

1$\alpha$,25-Dihydroxyvitamin D3 (vitD3)[3] is a seco-steroid hormone that is not only involved in mineral and skeletal homeostasis, but also regulates the differentiation, growth, and function of a broad range of cells, including cells of the immune system (20). VitD3 acts through its receptor, the nuclear vitD3 receptor, which in turn acts as a transcription factor by binding to distinct vitD3 receptor responsive elements that are present in the promoter regions of target genes. VitD3 also modulates gene transcription by affecting the activity of transcription factors, such as the NF-AT, NF-$\kappa$B, and SMAD families of transcription factors (21–23). Activated macrophages possess the enzyme 1-$\alpha$-hydroxylase that allows for the production of vitD3, which suggests a role for this endogenously produced steroid hormone in regulation of immune responses (24). Furthermore, vitD3 has been demonstrated to prevent Th1-mediated autoimmune diseases in animal models for experimental allergic encephalomyelitis, systemic lupus erythematosus, and type I diabetes (25–27). A number of studies have demonstrated that vitD3 modulates the activity of monocytes/macrophages and dendritic cells (16, 28–32). It was found that vitD3 inhibits the differentiation and maturation of DC; reduces the expression of MHC class II, CD40, CD80, and CD86; and inhibits the secretion of IL-1, IL-6, TNF-$\alpha$, and IL-12 (30–32). In vitro stimulation in the presence of APC, showed a vitD3-induced reduction of IFN-$\gamma$ production, whereas the Th2 compartment was

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[3] Abbreviation used in this paper: vitD3, 1$\alpha$,25-dihydroxyvitamin D3.



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not affected (26, 33, 34). In vivo administration of vitD3 resulted in an increase in IL-4 expression (35) and production (36), with a concomitant reduction of IFN-$\gamma$ levels. The inhibition of Th1 development by vitD3 is thought to be primarily mediated via its action on the APC as a consequence of reduced IL-12 production after vitD3 treatment (31).

These studies suggest that the vitD3-induced switch from Th1 to Th2 may be attributable to its ability to modulate the APC. However, it is unclear to date whether the effects of vitD3 on Th cell development are mediated solely via effects on the APC (e.g., reduced IL-12) or whether $CD4^+$ cells are also direct targets. It has been shown that vitD3 can act directly on T cells by inhibiting T cell proliferation (37). However, no studies have yet addressed whether vitD3 has any direct effects on $CD4^+$ T cells to affect their subsequent development into Th1 and Th2 cells. In this study we describe that vitD3 has a direct effect on $CD4^+$ T cells to enhance the development of Th2 cells in the absence of APC, producing IL-4, IL-5, and IL-10, and to augment the expression of GATA-3 and c-*maf*.

## Materials and Methods

### *Mice*

DO.11.10 mice transgenic for an $OVA_{323–339}$-specific $\alpha\beta$TCR were used as a source of Ag-specific T cells (38). BALB/cAnN (Taconic Farms, Germantown, NY) and C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were used to provide splenic APCs or T cells. All mice were housed under specific pathogen-free conditions and were used between 8 and 12 wk of age.

### *Reagents*

mAb used in culture were anti-IL-4 (11B11) (39), anti-IL-12 (clone C17.8.20, a kind gift of G. Trinchieri, Ref. 40), and anti-IFN-$\gamma$ (XMG 1.2, J. Abrams, DNAX, Palo Alto, CA). IL-2 and IL-4 were obtained from DNAX. IL-12 was purchased from R&D Systems (Minneapolis, MN). Anti-mouse CD3 (145-2C11) and anti-CD28 (37.51) mAb used for T cell stimulation were purchased from BD PharMingen (San Diego, CA). mAb used for T cell preparation were anti-B220, anti-CD8$\alpha$, anti-Mac-1, anti-CD4-FITC, and anti-L-selectin-PE (all mouse specific, BD PharMingen). mAb used for intracellular staining were anti-IFN-$\gamma$ (clone XMG1.2), anti-IL-4 (11B11), anti-IL-10 (JES5-16E3), anti-IL-5 (TRFK-5) and isotype controls (all from BD PharMingen).

Tissue culture medium used was RPMI 1640 (J.R. Scientific, Woodland, CA) supplemented with 10% FCS (heat inactivated for 30 min at 56°C, J.R. Scientific), 0.05 mM 2-ME (Sigma, St. Louis, MO), 10 mM HEPES buffer (Life Technologies, Grand Island, NY), 100 U/ml penicillin (Life Technologies) and 100 $\mu$g/ml streptomycin (Life Technologies), 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and 1 mM sodium pyruvate (BioWhittaker). $1,25(OH)_2$-vitamin D3 was purchased from Biomol (Plymouth Meeting, PA). OVA peptide was used as a synthetic peptide encoding chicken $OVA_{323–339}$ (Biosynthesis, Lewisville, TX).

### *Preparation of T cells and APC*

$CD4^+$ T cells were enriched by negative depletion using a mixture of anti-CD8$\alpha$, anti-B220, and anti-Mac-1 mAb, followed by goat-anti-rat Ig coated beads (Biomag; Polysciences, Warrington, PA) as described previously (13). Enriched $CD4^+$ T cells were then further purified using a FACStar$^{Plus}$ flow cytometer (BD Biosciences, San Jose, CA) to achieve >99% naive $CD4^+$ T cells on the basis of bright Mel-14, CD4 staining (41). Staining did not alter the function of the T cells (data not shown).

### *Stimulation of transgenic $CD4^+$ T cells for cytokine production*

Primary stimulations of $CD4^+$ T cells ($2.5 \times 10^5$/well) were conducted using OVA peptide (0.6 $\mu$M) and RBC-lysed spleen cells ($5 \times 10^6$/well, 3000 rad) as APC in a total volume of 2 ml in 24-well plates. In some cases, Ag and APC were replaced by cross-linked anti-CD3 (10 $\mu$g/ml) and soluble anti-CD28 (1 $\mu$g/ml) mAb, and $1.5 \times 10^6$ cells were used per 2-ml well in 24-well plates. Cultures received only medium (i.e., neutral) or vitD3 ($4 \times 10^{-8}$ M). In addition, some cultures were supplemented with anti-IL-4, anti-IL-12, or anti-IFN-$\gamma$ mAb (10 $\mu$g/ml) to block endogenously produced cytokines. The dose of vitD3 is in the range of that used in other studies (18, 30, 31) and did not induce apoptosis or strong inhibition of Th cell proliferation. Cells were split on day 3, and harvested on day 7, washed twice, counted, and restimulated as described for the primary stimulation with the addition of 10 ng/ml IL-2. Restimulation of the cells in the APC-free system was with 2 $\mu$g/ml anti-CD3, 2 $\mu$g/ml anti-CD28 mAb, and 10 ng/ml IL-2.

### *Cytokine assays*

IL-4 and IFN-$\gamma$ sandwich ELISA assays were performed as previously described (42). The method for intracellular cytokine staining was as reported (43). Briefly, cultured $CD4^+$ cells were stimulated with 10 ng/ml IL-2, 1 $\mu$g/ml PMA (Sigma), and 2 $\mu$g/ml ionomycin (Sigma) at $10^6$ cells/ml for 2 h at 37°C. After 2 h, 10 $\mu$g/ml brefeldin A (Epicentre Technologies, Madison, WI) was added and the cells were incubated for another 2 h. The cells were then fixed with 2% formaldehyde, permeabilized with 0.5% saponin, and stained as described before (43). Samples were analyzed on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

### *RNase protection assay*

RNA was prepared using the Rnazol B method (Cinna/Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions, and 7 $\mu$g RNA was used to perform the RNase protection assay. Riboprobes of GATA-3, T-bet, and 18S inserted in the pGEM vector were prepared by linearization with *Hin*dIII. c-*maf*, inserted in the pBSK vector, was linearized using *Bgl*II before use in the RNase protection assay. The expected band size was as follows. GATA-3, 418 bp; c-*maf*, 329 bp; T-bet, 290 bp; and 18S, 187 bp. The probes (0.1 $\mu$g each) were labeled with $^{32}P$ using an in vitro transcription kit (BD PharMingen). Samples were run on a 5% acrylamide gel and were visualized using phosphorimaging (Molecular Dynamics, Sunnyvale, CA). Transcript levels were quantified using ImageQuant software (Molecular Dynamics).

## Results

### *VitD3 enhances Th2 cell development in the presence of APC*

To address the effect of vitD3 on Th cell development we cultured naive TCR-transgenic $CD4^+$ DO11.10 T cells with splenic APC and OVA peptide under neutral conditions (medium alone, no additions) or medium supplemented with vitD3. After two rounds of stimulation, the cytokine profile for intracellular cytokine production was determined by flow cytometry upon restimulation. As depicted in Fig. 1*A*, vitD3-driven Th cells showed an increased frequency of IL-4-producing cells and a reduction in the number of IFN-$\gamma$-producing cells as compared with cells grown under neutral conditions. Consistent with augmented Th2 development after culture with vitD3, an increase in IL-10- and IL-5-producing Th cells was also detected. Culture of Th cells under Th2 (IL-4/anti-IL-12) polarizing conditions resulted in a more pronounced Th2 phenotype (55% IL-4-producing cells) as compared with cells cultured with vitD3 (41% IL-4-producing cells). As shown in Fig. 1*B*, the effect of vitD3 on the cytokine production reflects augmented Th2 development with reduced IFN-$\gamma$ and increased IL-4 production, which is in accordance with the cytokine profile as determined by flow cytometry. Although the effects of vitD3 on Th development were more pronounced after repeated stimulation, a single stimulation was sufficient to demonstrate enhanced development of Th2 cells by vitD3 (Fig. 1*C*).

### *VitD3-induced Th2 development is dependent on IL-4*

Because IL-4 is the major cytokine that directs the development of Th2 cells, we determined whether IL-4 played a role in the observed augmented development of Th2 cells after in vitro culture in the presence of vitD3. To examine this we included cultures in which IL-4 was neutralized during the entire culture period. Fig. 2 shows that addition of anti-IL-4 mAb to the cultures containing vitD3 completely abrogated the development of Th2 cells producing IL-4. In fact, when IL-4 was neutralized, the frequency of IL-4-producing cells cultured with vitD3 was similar to that observed when cells were cultured under neutral conditions in the presence of anti-IL-4 mAb (Fig. 2). These data indicate that IL-4

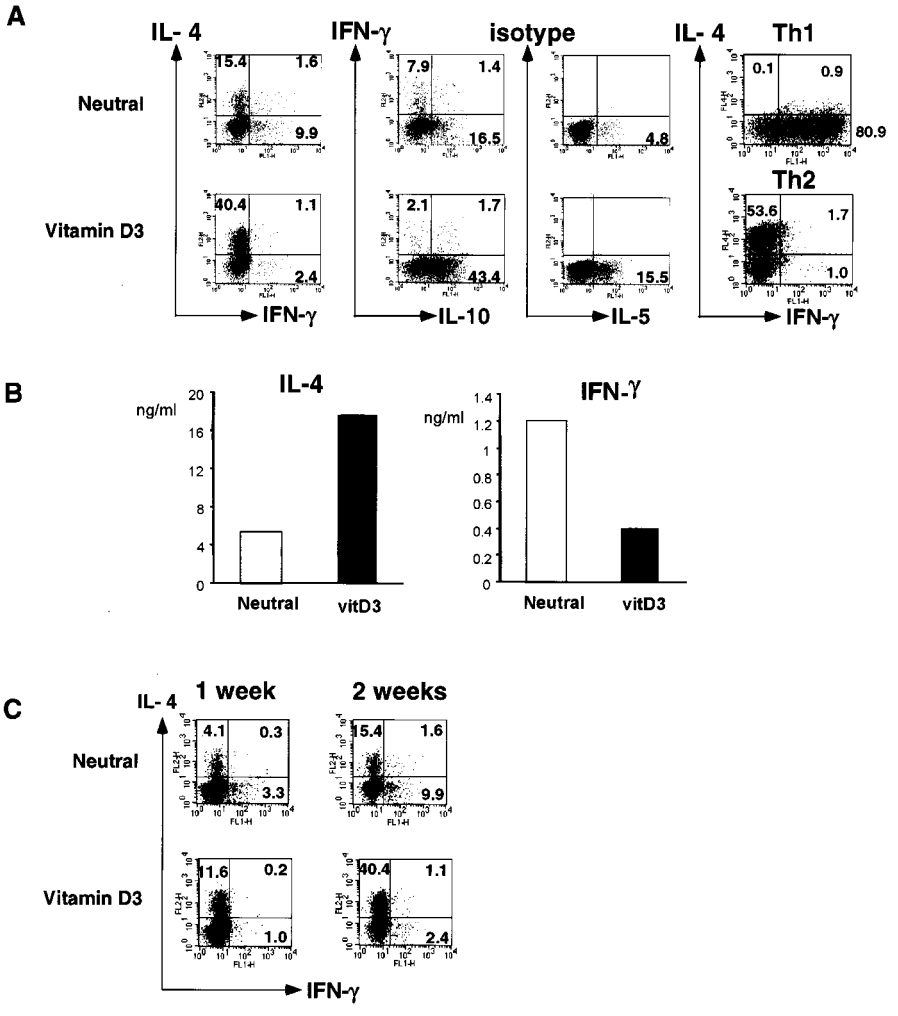


**FIGURE 1.** VitD3 enhances Th2 cell development in the presence of APC. $CD4^+Mel14^+$ DO11.10 T cells were activated with $OVA_{323–339}$ peptide and γ-irradiated splenic APC. The cells were stimulated with or without (neutral) the addition of vitD3, or with IL-12/anti-IL-4 (Th1 control), or IL-4/anti-IL-12 (Th2 control). After two rounds of stimulation, cells were harvested and restimulated with PMA/ionomycin for cytokine production by intracellular cytokine staining for 4 h (*A*) or immunoassay for 48 h (*B*). *C*, The cytokine profile after one and two rounds of stimulation.

is required for the vitD3-induced effects on Th cells development. Addition of anti-IL-4 mAb resulted in a significant increase in the frequency of IFN-γ-producing cells.

### *VitD3 has a direct effect on Th cells resulting in strong polarization toward a Th2 profile*

A number of studies have reported vitD3-induced reduction of IL-2 and IFN-γ production, but only in some cases a subsequent enhancement of Th2-type cytokines by T cells in the presence of APC (18, 33, 44). To determine whether vitD3 also has a direct effect on Th cell development independently of APC related effects we activated naive $CD4^+$ $Mel14^+$ T cells with plate bound anti-CD3 and soluble anti-CD28 mAb in the presence or absence of vitD3. The cells were repeatedly stimulated, and their cytokine profile was assessed after 3 wk in culture. Fig. 3*A* shows that purified naive $CD4^+$ $Mel14^+$ T cells obtained from BALB/c mice stimulated in the absence of APC develop into a highly polarized Th2 population when cultured with vitD3, similarly to APC-driven cultures. The frequency of IL-4-producing cells was enhanced from 8.0 to 55.8%. In accordance with this, the frequency of IL-10- and IL-5-producing cells was also dramatically increased, whereas the frequency of IFN-γ-producing cells was reduced. The frequency of IL-4-producing cells after culture with vitD3 was similar to cultures with Th cells under Th2 (IL-4/anti-IL-12) polarizing conditions (55.8 and 58.6%, respectively). To examine whether the effects of vitD3 are limited to the BALB/c genetic background or more generalized, we stimulated $CD4^+Mel14^+$ T cells from C57BL/6 mice with anti-CD3/anti-CD28 in the presence of vitD3. As shown in Fig. 3*B* Th cells from C57BL/6 mice also develop into highly polarized Th2 cells after single or multiple rounds of stimulation. These findings clearly indicate that Th cells can be direct targets for the effects of vitD3 on Th development as demonstrated by significant induction of Th2 polarization in both BALB/c and C57BL/6 mice.

### *Neutralization of IL-4 abrogates vitD3-induced Th2 polarization independently of IFN-γ*

The enhanced vitD3-driven Th2 development was also abolished in these APC-free cultures by neutralization of IL-4 (Fig. 4). However, neutralization of IL-4 also led to an increase in the percentage of IFN-γ-producing cells. Thus it was possible that overproduction of IFN-γ, which can inhibit Th2 development (45), could be responsible for reduced numbers of IL-4-producing cells, rather than a requirement for IL-4 in vitD3-driven Th2 development. To exclude this possibility we simultaneously neutralized both IL-4 and IFN-γ and assessed the cytokine profile of naive $CD4^+$ cells after culture in the presence or absence of vitD3. Cells were repeatedly stimulated with cross-linked anti-CD3 and soluble anti-CD28 mAb. As depicted in Fig. 4, when anti-IL-4 and anti-IFN-γ mAb were added throughout the culture period of vitD3-driven cultures, the cytokine profile of these vitD3 cultured cells resembled that of cells cultured under neutral conditions (with no blocking mAb) with a low frequency of IL-4-producing cells in vitD3-driven cultures when IL-4 and IFN-γ were absent. Taken together, these data

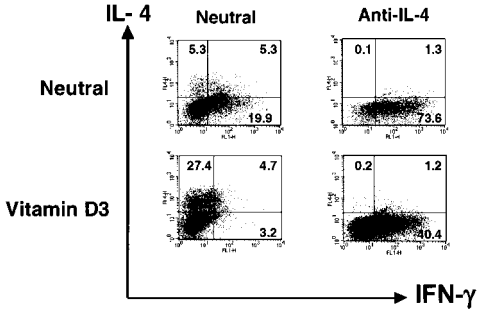


**FIGURE 2.** IL-4 dependence of vitD3 induced Th2 development when cultured in the presence of APC. $CD4^+Mel14^+$ DO11.10 T cells were activated with $OVA_{323–339}$ peptide and γ-irradiated splenic APC. Cultures were performed with and without the addition of vitD3, as well as with or without the addition of anti-IL-4 mAb. After three rounds of stimulation the cells were restimulated with PMA/ionomycin and the cytokine profile was determined by flow cytometry. *A*, The staining for IFN-γ and IL-4; *B*, IFN-γ and IL-10 staining.

clearly demonstrate that the effects of vitD3 on the development of $CD4^+$ T cells are largely dependent on IL-4, although small numbers of IL-4-producing cells can develop in the complete absence of IL-4.

### *VitD3 up-regulates the expression of GATA-3 and c-*maf *in developing Th cells*

In recent years considerable progress has been made in the knowledge of transcription factors involved in Th1 and Th2 cell differentiation. The transcription factor T-bet has been described to direct the differentiation and commitment of Th1 cells, and to induce IFN-γ expression (14). c-*maf* and GATA-3 are Th2-specific transcription factors that regulate the Th2 cytokine genes (8–13). We examined whether vitD3 cultured Th cells showed a transcription factor pattern reflecting the augmented Th2 development as indicate by their cytokine profile. As shown in Fig. 5 and in accordance with previously published findings, highly polarized Th1 cells obtained after repeated stimulation expressed T-bet, but low to undetectable levels of c-*maf* and GATA-3. Conversely, Th2 cells expressed GATA-3 and c-*maf*, but not T-bet. Naive $CD4^+Mel14^+$ T cells stimulated with anti-CD3 and anti-CD28 mAb in the presence of vitD3 showed enhanced levels of GATA-3 and c-*maf* expression as compared with cells grown under neutral conditions. No T-bet expression was detected in cells cultured under neutral or vitD3-driven conditions. These findings are representative of two independent experiments. Therefore, the transcription factor profile correlates with the increased Th2 cytokine production of $CD4^+$ cells cultured in the presence of vitD3.

## Discussion

In the present study we demonstrated that vitD3 affects the Th1-Th2 balance in that it inhibits Th1 and augments Th2 cell development. These effects were observed when T cells were stimulated with anti-CD3/anti-CD28 alone, as well as when stimulated with APC and Ag, indicating that $CD4^+$ cells themselves can be direct targets for vitD3 resulting in enhanced Th2 development. The increased production of Th2 cytokines correlated with increased expression of the Th2-specific transcription factors GATA-3 and c-*maf* after vitD3 treatment in vitro. Furthermore, we determined IL-4 to be a major mediator of the enhanced vitD3 induced Th2 development, although small numbers of IL-4-producing cells were induced in the absence of IL-4 and IFN-γ.

It has been suggested that the immunomodulatory effects of vitD3 are mainly mediated via modulation of the activity of macrophages and dendritic cells, via inhibition of their differentiation, maturation, and T cell-stimulatory activity (30, 31, 46). More specifically, reduced expression of costimulatory molecules (CD80, CD86, CD58, and CD40) and inhibition of the production of cytokines, like TNF-α, IL-6, and IL-12, were shown to result in impaired T cell activation (30). The inhibitory effect of vitD3 on

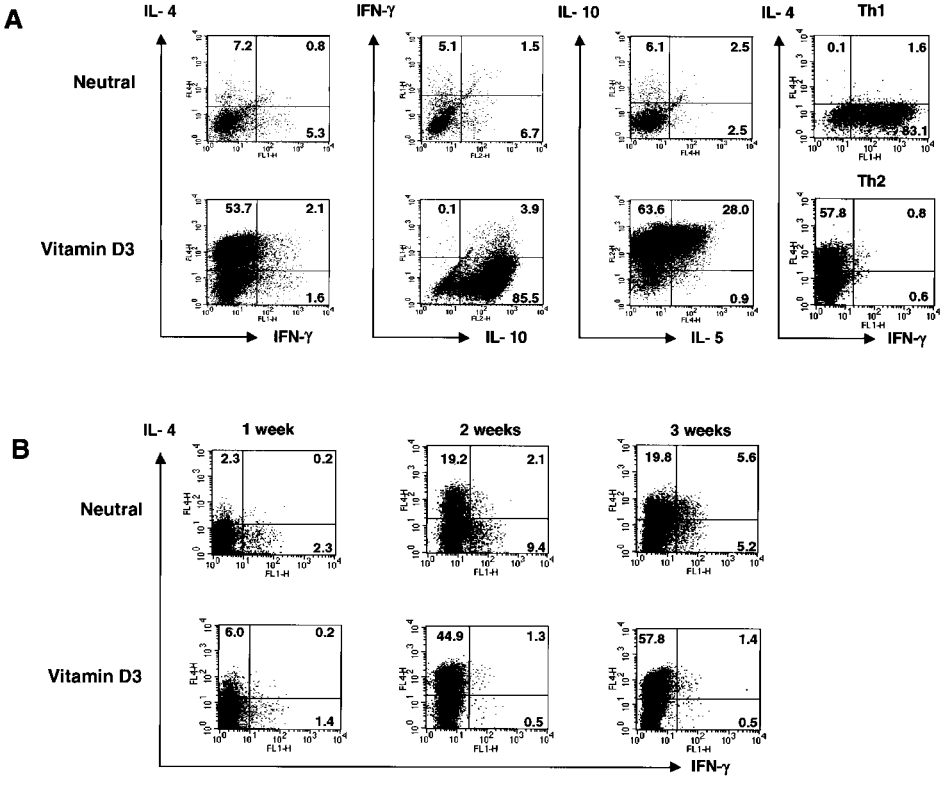


**FIGURE 3.** Enhanced vitD3-induced Th2 development occurs in the absence of APC. $CD4^+$ $Mel14^+$ T cells from BALB/c mice were activated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence or absence of vitD3, or with IL-12/anti-IL-4 (Th1 control), or IL-4/anti-IL-12/anti-IFN-γ (Th2 control). Cells were restimulated three times and the cytokine profile was determined after restimulation with PMA/ionomycin and assessed by flow cytometry (*A*). Identical experiments were performed with $CD4^+Mel14^+$ T cells obtained from C57BL/6 mice, and their cytokine profile was determined after one, two, and three rounds of stimulation (*B*).

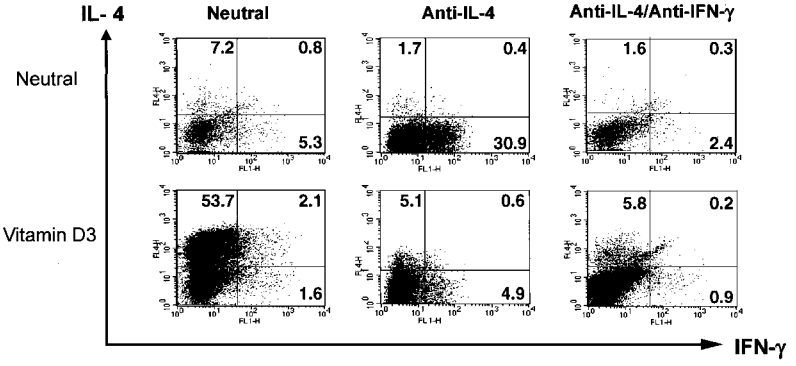


**FIGURE 4.** Neutralization of IFN-γ and IL-4 simultaneously compensates for the vitD3 induced effects on Th cell development. $CD4^+Mel14^+$ T cells from BALB/c mice were activated as described in Fig. 3. Additionally, cultures were included containing anti-IL-4 alone, or a mixture of anti-IL-4, anti-IFN-γ mAb, and anti-IL-12 mAb.

IL-12p70 production by DC via inhibition of NF-κB activation and transcriptional repression of the IL-12p40 gene (31) is likely to play a dominant role in the observed reduction of Th1 cell development after treatment with vitD3.

Although APC can be direct targets for the immunomodulatory activities of vitD3, we clearly show that vitD3 also has a direct effect on the differentiation of naive $CD4^+Mel14^+$ T cells upon TCR stimulation, resulting in inhibition of Th1 and augmented Th2 development. The vitD3 induced skewing toward a Th2 phenotype was much more pronounced when $CD4^+Mel14^+$ T cells were activated with anti-CD3/anti-CD28 mAb as compared with cells activated in the presence of APC and Ag. It is possible that APC may deliver signals to inhibit Th2 development, such as via IL-12 (31) or ICAM-1 (47, 48) which counteract the effects of vitD3. Additionally by its effect on downstream signaling pathways known to be triggered by TCR ligation, it is possible that vitD3 may modify the strength of the signal delivered to the T cell which has been shown to affect the development of Th1 and Th2 responses (49).

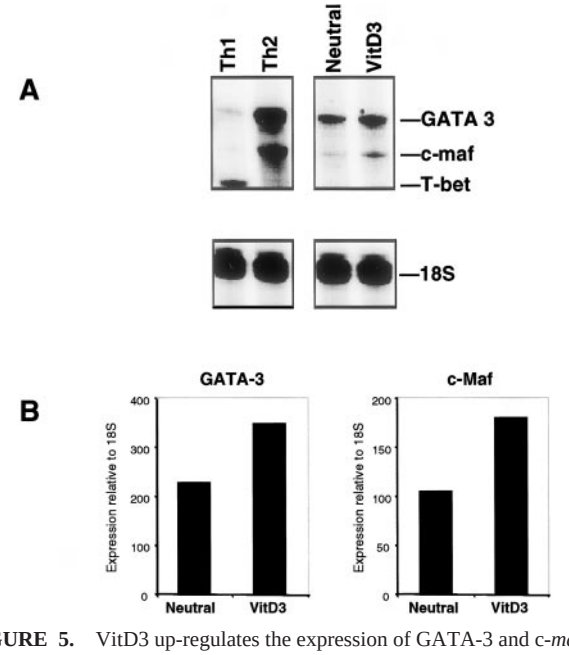


**FIGURE 5.** VitD3 up-regulates the expression of GATA-3 and c-*maf* in developing Th cells. *A*, RNase protection assay for GATA-3, c-*maf*, T-bet, and 18s transcripts was performed using total cellular RNA of $CD4^+Mel14^+$ cells stimulated in the presence or absence of vitD3. RNA from cells cultured at Th1 (IL-12, anti-IL-4) or Th2 (IL-4, anti-IL-12, anti-IFN-γ) priming conditions are included as positive controls. *B*, Expression of GATA-3 and c-*maf* as determined by densitometry. Values are expressed in arbitrary units relative to the 18s signal. Values are representative of two independent experiments.

It has been reported that the transactivation of the IFN-γ promoter is down-regulated by vitD3 in transfected Jurkat cells (50). Thus, in our system, it is possible that transcriptional suppression of the IFN-γ gene takes place, thereby providing a possible explanation for the suppression of IFN-γ production. This can be augmented by up-regulation of GATA-3 seen in the presence of vitD3, because GATA-3 has been shown to inhibit the development of IFN-γ-producing cells early during Th cell differentiation (11, 13). To date no vitD3 receptor responsive elements in the IL-4 promoter have been described, but the ability of vitD3 to up-regulate c-*maf* expression could account for increased transactivation of the IL-4 promoter.

The vitD3 induced effects on Th development were largely mediated via IL-4. However, a small population of IL-4-producing cells was detected after culture in the presence of vitD3 in the presence of neutralizing mAb to IFN-γ and IL-4. This IL-4-independent Th2 cell development may be reminiscent of the findings by Ouyang et al. (51), who showed STAT6-independent Th2 development.

We showed that Ag-specific stimulation of $CD4^+$ T cells in the presence of vitD3 inhibits the development of Th1 cells. Conversely, we demonstrated the development of highly Th2 skewed populations after in vitro culture with vitD3 with increased IL-4, IL-5, and IL-10 production as determined by the intracellular cytokine profile and immunoassay. The presence of vitD3 is required during each phase of stimulation, because the developing Th2 cells are still unstable until they are committed. Even Th cells cultured with IL-12/anti-IL-4 mAb or IL-4/anti-IL-12 require these additives until they are committed to the Th1 or Th2 phenotype, respectively (7). Additionally, we provide data that vitD3 induced Th2 development can also be seen not only on the BALB/c but also on the C57BL/6 genetic background. The vitD3 induced inhibition of Th1 development in APC/Ag stimulated cultures has been described before (18, 33); therefore, our data confirm these findings. However, the vitD3 induced increase of Th2 development has not been demonstrated in vitro, and appears in apparent conflict with previous studies where effects on Th2 development were minimal. In contrast to other studies (18, 33), we used naive $CD4^+$ T cells and assessed the role of vitD3 in the well-defined OVA-TCR transgenic system. Mattner et al. (33) showed the effect of vitD3 on human cord blood $CD4^+CD45RA^+$ T cells after stimulation autologous adherent cells and PHA. After 10 days in culture they found a reduction of IFN-γ production but only a mild increase of IL-4. Differences may thus be explained by the different source of naive T cells, and it is also possible that there is donor variation in humans.

The physiological role of vitD3 in immune responses is still not defined. Macrophages have been shown to synthesize vitD3 when activated with LPS and IFN-γ (52) (data not shown). This implies that upon triggering of APC by microbial factors vitD3 is synthesized by these cells and thus may down-regulate the production of inflammatory mediators, such as IL-12. This could initiate a cascade of events resulting in reduced APC function and polarization of Th cells toward Th2 dominated responses. The suppression of IFN-γ production might prevent a sustained synthesis of vitD3, thereby providing a negative feedback mechanism. Therefore, it is likely that vitD3 functions to dampen cell-mediated immune responses through prevention of strong Th1 responses via the action on the APC, while simultaneously directly acting on the T cell to enhance Th2 cell development. VitD3 is also synthesized by keratinocytes after exposure of the skin to ultraviolet B irradiation. Little is known about the role of locally produced vitD3 in the skin. However, it is tempting to speculate that vitD3 contributes to the immunomodulatory effects of ultraviolet B by affecting the activity of APC and Th cells.

The dose of vitD3 used in our study was in the range of that used by others and did not induce apoptosis or strong inhibition of Th proliferation. Because macrophages, dendritic cells, and keratinocytes can produce vitD3 it is likely that at the localized level in vivo, high vitD3 can be found which may exert its effect on neighboring Th cells. For these reasons, however, it may be difficult to extrapolate from physiologically relevant concentrations of vitD3 in vivo to the in vitro system we are using.

In this study we have provided insight into the effect of vitD3 on Th polarization by showing for the first time that vitD3 is able to enhance Th2 development in the absence of APC. The potent direct effect of vitD3 on Th cells may, at least in part, explain its beneficial effects in the treatment of psoriatic lesions as well as a number of other autoimmune diseases. Augmented immune deviation into strong Th2 mediated responses by combined effects on APC and Th cells are likely to underlie the immunosuppressive activity of vitD3.

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