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1,25-Dihydroxyvitamin D_3 Inhibits IFN-γ and IL-4 Levels During In Vitro Polarization of Primary Murine $CD4^+$ T Cells[1]

Teodora P. Staeva-Vieira* and Leonard P. Freedman[2†]

Following their activation, naive $CD4^+$ T cells can differentiate into one of two effector cell subsets, Th1 and Th2. These two subsets have different cytokine secretion patterns and thus mediate separate arms of the immune response. It has been established that the fat-soluble vitamin D_3 metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its nuclear receptor, the vitamin D receptor, play an important role in the immune system primarily through the transcriptional inhibition of cytokine genes that either are required for Th1 differentiation or are products of differentiated Th1 cells. Therefore, we wanted to test directly the ability of $1,25(OH)_2D_3$ to alter the Th differentiation process. Our results indicate that $1,25(OH)_2D_3$ inhibits not only the Th1 cytokine IFN-γ but also the Th2 cytokine IL-4 in naive CD62 ligand$^+$$CD4^+$ T cells during their in vitro polarization. This effect is most dramatic when the ligand is present from the onset of the differentiation process. If the ligand is added after the polarization has ensued, the inhibition is significantly diminished. In activated (CD62 ligand$^-$$CD4^+$) T cells, $1,25(OH)_2D_3$ is still able to inhibit IFN-γ but has no effect on IL-4 production. Our results also indicate that inhibition of these two cytokines in naive cells by vitamin D receptor and its ligand is neither a result of a cell cycle block nor an inhibition of Th1 or Th2 transcription factor expression but, rather, at least in the case of Th2 differentiation, an attenuation of IL-4 transcription by the receptor. ***The Journal of Immunology*****, 2002, 168: 1181–1189.**

Upon Ag stimulation, naive $CD4^+$ Th cells differentiate into two distinct effector populations, Th1 and Th2, based on their cytokine secretion patterns (1). One of the most crucial factors in the polarization process is the cytokine environment. Thus, in vitro, Th1 and Th2 subsets can be generated by activating naive $CD4^+$ T cells in the presence of IL-12 or IL-4, respectively. IL-12 activates the Stat4 signaling pathway to induce IFN-γ transcription and initiate Th1 differentiation, while IL-4 triggers the Stat6 signaling pathway, resulting in IL-4 transcription and Th2 differentiation (2). Once established, the two subsets have distinct cytokine secretion profiles that not only serve to define them (with IFN-γ being the Th1 signature cytokine while IL-4 is the defining Th2 cytokine) but also endow them with different functional properties. 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. In addition to the cytokine environment, many other factors have been shown to play an important role in the differentiation process. Some of these include the Th1- and Th2-specific transcription factors, the strength of TCR signaling, the Ag dose, costimulatory signals, and the influence of the APCs (3–7). Furthermore, some less conventional factors, such as PGE_2 (8, 9) and the steroids progesterone (10) and dexamethasone (reviewed in Ref. 11), have also been implicated in influencing the differentiation process. Our studies focus on the role of another secosteroid, vitamin D_3, in Th differentiation.

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[3] the active metabolite of vitamin D_3, is a lipophilic molecule which exerts its actions through a nuclear receptor, the vitamin D receptor (VDR) (reviewed in Refs. 12 and 13). VDR is a member of the steroid-nuclear receptor superfamily, whose members include receptors that bind glucocorticoids, retinoids, thyroid hormones, sex steroids, fatty acids, and eicosanoids (14). In the presence of its ligand, VDR, together with its heterodimeric partner the retinoid X receptor (RXR), can activate or repress target genes by binding to vitamin D response elements on DNA (15, 16). Although traditionally $1,25(OH)_2D_3$ has been associated with regulating calcium homeostasis, the discovery of VDR expression in lymphocytes and monocytes (17–19) suggested a role for this hormone in the immune system as well. Indeed, a number of studies have now demonstrated the ability of this receptor-ligand pair to act as a strong immunosuppressor (reviewed in Refs. 20 and 21). In promonocytes, $1,25(OH)_2D_3$ has been shown to exert strong antiproliferative and prodifferentiation properties (reviewed in Ref. 22). In contrast, in dendritic cells this hormone inhibits differentiation, maturation, and activation in both human and mouse cells (23–25). In T lymphocytes, $1,25(OH)_2D_3$ also diminishes proliferation (26–28), most likely by virtue of its ability to inhibit IL-2 transcription (29). Thus far, $1,25(OH)_2D_3$ and its receptor have been shown to down-modulate the production of other key cytokines, such as IFN-γ (30–32), IL-12 (33), and GM-CSF (34, 35).

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[3] Abbreviations used in this paper: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; RXR, retinoid X receptor; EAE, experimental autoimmune encephalomyelitis; CD62L, CD62 ligand.

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The effects of $1,25(OH)_2D_3$ on the immune system are often inhibitory and frequently result from binding of VDR to nonconsensus vitamin D response elements (29, 35, 36). Furthermore, the mechanisms of the repression on all of the above mentioned gene promoters caused by $1,25(OH)_2D_3$ have been described (36–38). Our laboratory first demonstrated what now appears to be a common repressive mechanism of VDR and its ligand in the immune system (29, 39). Specifically, VDR interferes with binding of NFAT/AP-1 to key regulatory sites in the promoters of many of the cytokine genes, resulting in a repression of activated transcription of such genes (29, 36, 38).

From the above mentioned studies, it is apparent that $1,25(OH)_2D_3$ inhibits cytokines which either are required for Th1 differentiation, such as IL-12, or are products of differentiated Th1 cells (IL-2 and IFN-γ), suggesting that one of the functions of $1,25(OH)_2D_3$ in the immune system is to inhibit Th1 differentiation (33, 40). Before initiating our studies, a considerable body of evidence existed for the inhibitory effects of $1,25(OH)_2D_3$ on Th1 differentiation. However, not much was known about the possible role this hormone may play in Th2 polarization. Given the established role of $1,25(OH)_2D_3$ as an immunoregulator, we decided to study the possible effect VDR may have during Th differentiation. Our studies indicate that $1,25(OH)_2D_3$ inhibits not only Th1 but also Th2 differentiation during in vitro polarization of naive $CD4^+$ T cells. This inhibition is dependent on the presence of the hormone during the initial stages of Th differentiation and seems to be mediated most likely via a mechanism of transcriptional repression by VDR of the key Th1 and Th2 cytokines IFN-γ and IL-4, respectively. Our results suggest that $1,25(OH)_2D_3$ acts as another influence on the differentiation of the Th1 and Th2 subsets. Thus, levels of $1,25(OH)_2D_3$ at sites of inflammation may have a significant effect on the initiation of the immune response.

Materials and Methods

Mice, cytokines, and Abs

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). IL-2 was purchased from BioSource International (Camarillo, CA), IL-4 was purchased from Endogen (Woburn, MA), IL-12 was purchased from R&D Systems (Minneapolis, MN), and anti-IL-4, anti-IL-12, anti-CD3, anti-CD28, anti-CD62 ligand (CD62L)-PE, and all other fluorochrome-conjugated Abs were purchased from BD PharMingen (San Diego, CA). All ELISA and ELISPOT Abs were purchased from Endogen.

Preparation of $CD4^+$ T cells

Splenocytes from 6- to 8-wk-old BALB/c mice were purified by RBC lysis. $CD4^+$ T cells were isolated by positive selection using CD4 Dynabeads followed by detachment with DETACHaBEAD (Dynal Biotech, Oslo, Norway). The purified $CD4^+$ T cells were labeled with R-PE-conjugated anti-CD62L Ab and sorted into $CD62L^+$ and $CD62L^-$ using a MoFlo (Cytomation, Fort Collins, CO) cell sorter. The sorted cells were pelleted at 1200 rpm at 4°C, resuspended in complete medium, and used in in vitro differentiation experiments. The complete medium consisted of RPMI supplemented with 10% FBS plus penicillin-streptomycin, sodium pyruvate, and glutamine to a final concentration of 100 μg/ml. In addition, 2-ME (Sigma-Aldrich, St. Louis, MO) was added to final concentration of 50 μM and gentamicin (Life Technologies, Grand Island, NY) to 50 ng/ml.

In vitro differentiation of $CD4^+$ T cells

$CD62L^+$ or $CD62L^-$ cells (at 0.5–1 $\times$ 10^6 cells/ml) were stimulated in vitro with plate-bound anti-CD3 mAb (2C11) at 3 μg/ml and anti-CD28 mAb (37.51) at 1 μg/ml plus IL-2 at 25 U/ml (nonskewing condition). In addition, cells were incubated with IL-12 at 5 ng/ml and anti-IL-4 mAb (11B11) at 3 μg/ml (Th1-skewing condition) or IL-4 at 500 U/ml and anti-IL-12 (C17.8) at 6 μg/ml (Th2-skewing condition). Differentiation proceeded in the presence of 2.4×10^{-8} M $1,25(OH)_2D_3$ (a kind gift of M. Uskokovic, Hoffmann-LaRoche, Nutley, NJ) diluted in ethanol or in the presence of an equal volume of ethanol only. Four days poststimulation, the cultures were expanded 4-fold with fresh medium, cytokines, and Abs. Three days later, the cells were harvested, washed five times with RPMI 1640 and counted, and equal number of cells were restimulated with plate-bound anti-CD3 at 1 μg/ml. Twenty-four hours postrestimulation, supernatants were collected and cytokine levels were measured by ELISA.

FACS analysis

Cell staining for CD3, CD4, and CD8 was performed by incubating 50 μl of cell suspension ($\sim$1 $\times$ 10^6 cells) with 0.2 μg of the appropriate Ab (anti-CD3 CyChrome, anti-CD4 FITC, anti-CD8 PE) for 30 min on ice. The cells were washed, resuspended in PBS, and analyzed using a FACSCalibur.

IL-4 and IFN-γ ELISA

Ninety-six-well Nunc ELISA MaxiSorp flat-bottom plates (Fisher Scientific, Pittsburgh, PA) were coated overnight at room temperature with 1 μg/ml IL-4 coating mAb in PBS (pH 7.4) or with 0.5 μg/ml coating IFN-γ mAb in coating buffer B (0.03 M sodium carbonate, 0.068 M sodium bicarbonate). The primary mAbs were discarded and the plates were blocked with 200 μl/well assay buffer (4% BSA in PBS (pH7.4)) for 1 h at room temperature. The plates were washed three times with wash buffer (50 mM Tris (pH 8), 0.2% Tween 20) and blotted on paper towel, and 50 μl/well of the standards or samples were added in duplicates. Some samples required prior dilution in complete medium to fall within the standard curve range. Following a 1-h incubation at room temperature, 50 μl/well of the detecting mAb was added: 100 ng/ml anti IL-4 secondary Ab or 250 ng/ml anti IFN-γ Ab. The plates were incubated for 1 h at room temperature and washed three times with wash buffer, and 100 μl/well HRP-conjugated streptavidin was added at 1/32,000 dilution for IL-4 or 1/8,000 dilution for IFN-γ in assay buffer. After a 30-min incubation at room temperature, the plates were washed as before and 100 μl/well tetramethylbenzidine substrate solution was added. The color was allowed to develop for 30 min in the dark before the reaction was quenched with 100 μl/well stop solution (0.18 M H_2SO_4). The plates were read at 450–550 nm and the sample concentrations were determined with the help of the standard curve. All reagents for the ELISAs were purchased from Endogen.

CFSE labeling and analysis

Murine $CD4^+$ T cells, isolated as described above, were further purified into $CD62L^+$ and $CD62L^-$ by positive isolation with detachment using a biotin-conjugated anti-CD62L mAb (BD PharMingen) and a CELLection Biotin Binder kit (Dynal Biotech). The purity of the isolated cells was confirmed by FACS using a PE-conjugated anti-CD62L mAb (BD PharMingen). The cells were subsequently resuspended in 1 ml of HBSS (at $\leq$ 2 $\times$ 10^7 cells/ml) and labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) at room temperature for 8 min. The reaction was quenched with 1 ml of FBS and the cells were washed four times with complete medium. After the final wash, the cells were allowed to rest overnight in complete medium at 37°C and 5% CO_2. A small aliquot of the labeled cells was analyzed by FACS for CFSE incorporation. The rest of the cells were pelleted, resuspended in 1 ml of complete medium, counted, diluted to 0.5–1 $\times$ 10^6 cells/ml, and activated on Ab-coated plates in the presence of the appropriate skewing cytokines and Abs as described above.

Cell culture and transfection

Jurkat, a human T cell lymphoma, was maintained in RPMI 1640 supplemented with 10% FBS plus penicillin-streptomycin, sodium pyruvate, and glutamine to a final concentration of 100 μg/ml. The cells were transfected by electroporation as previously described (38).

RT-PCR

Total RNA was isolated, according to the manufacturer's instructions, using TRIzol reagent (Life Technologies) from in vitro activated $CD4^+$ T cells. RNA (1.5 μg) was used in the reverse transcription reaction with Superscript II RNase H^- (Invitrogen, San Diego, CA) following the manufacturer's instructions. The cDNA was diluted 5-fold in water and 5 μl were amplified 25 cycles in PTC-200 Peltier Thermal Cycler (MJ Research, Cambridge, MA) in a reaction containing 5 μl of 10$\times$ PCR buffer with 15 mM $MgCl_2$, 1 μl of 20 mM sense and antisense primers, 1 μl of 10 mM dNTPs, 36.5 μl of water, and 0.5 μl of *Taq*. The cycling conditions were 94°C for 2 min followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 5 min. The following primers were used: GATA-3 sense, GAAGGCATCCAGAC CCGAAAC, and antisense, ACCCATGGCGGTGACCATGC; c-Maf sense, CCCAGTCCTGCCGCTTCAAGAGGG, and antisense, CATT GAACATTGTGCAAGTCC; T-bet sense, TGCCTGCAGTGCTTCTAACA, and antisense, TGCCCCGCTTCCTCTCCAACCAA, as published in Ref. 41;

IL-4 sense, CATCGGCATTTTGAACGAGGTCA, and antisense, CTTATC GATGAATCCAGGCATCG; and HPRT sense, GTTGGAGACAGGCCAG ACTTTGTTG, and antisense, GAGGGTAGGCTGGCCTATAGGCT. The IL-4 and HPRT primer sequences were as published in Ref. 42.

Results

1,25(OH)$_2$D$_3$ inhibits IFN-γ and IL-4 production during Th differentiation of naive CD62L$^+$CD4$^+$ T cells

To test directly whether 1,25(OH)$_2$D$_3$ can affect the Th polarization process, we conducted in vitro Th differentiation experiments. Briefly, naive (CD62$^+$) CD4$^+$ T cells (Fig. 1*A*) were isolated from spleens of BALB/c mice and were activated in culture under nonskewing, Th1-skewing, or Th2-skewing conditions in the absence (shaded bars) or presence (filled bars) of 1,25(OH)$_2$D$_3$. After 7 days, the cells were washed and restimulated for 24 h in the absence of exogenous cytokines and Abs. Supernatants were collected and levels of secreted IFN-γ and IL-4 were determined using an ELISA. As expected, we observed that 1,25(OH)$_2$D$_3$ inhibited IFN-γ production in naive T cells by at least 50% (Fig. 1*B*). Likewise, 1,25(OH)$_2$D$_3$ also inhibited IL-4 production, but only in cultures polarized toward the Th2 condition (Fig. 1*C*). Under nonpolarizing conditions, 1,25(OH)$_2$D$_3$ did not have an inhibitory effect on IL-4 production. In fact, the presence of the hormone enhanced IL-4 levels, 1.4 ng/ml with ligand as compared with 0.3 ng/ml for the vehicle control (Fig. 1*C*). Similar results were obtained using an ELISPOT assay (data not shown). Thus, 1,25(OH)$_2$D$_3$ appears to inhibit not only IFN-γ but also IL-4 production during the in vitro differentiation of naive CD4$^+$ T cells toward the Th1 and Th2 subsets, respectively.

1,25(OH)$_2$D$_3$ inhibits IFN-γ but not IL-4 production during Th differentiation in activated CD62L$^-$CD4$^+$ T cells

In addition to testing the effects of 1,25(OH)$_2$D$_3$ on naive CD4$^+$ T cells, we also wondered how this hormone would affect the in vitro Th differentiation of activated CD4$^+$ T cells. To test this, we set up the Th differentiation experiments previously described but using CD62L$^-$ T cells (Fig. 1*A*). Similar to its effects on naive cells, 1,25(OH)$_2$D$_3$ inhibited IFN-γ production in the CD62L$^-$ T cells (Fig. 2*A*). However, unlike its effect on naive cells, the hormone did not alter IL-4 production in this cell population (Fig. 2*B*). Similar results were obtained using the ELISPOT assay (data not shown). It should be noted that the unskewed population (Fig. 2*B*) produced as much IL-4 as the Th2-polarized cells. We believe this is due to the genetic background of the mice used, BALB/c, which develop preferentially a Th2 response. Our results suggest that in previously activated CD4$^+$ T cells, 1,25(OH)$_2$D$_3$ inhibits only IFN-γ production but is ineffective in altering IL-4 levels during the in vitro differentiation of these cells.

Temporal effects of 1,25(OH)$_2$D$_3$ on naive CD4$^+$ T cells

Because 1,25(OH)$_2$D$_3$ was able to inhibit IL-4 production in naive cells but was ineffective in suppressing this cytokine in previously activated T cells, we wondered at what time point after the Th differentiation process has begun can the ligand be first added and still be able to inhibit IL-4 production. Thus, we established the following experimental system (Fig. 3*A*): naive CD4$^+$ T cells were differentiated under polarizing or nonpolarizing conditions, as previously described, and 1,25(OH)$_2$D$_3$ was added at the beginning of the differentiation process (treatment 1), halfway through the differentiation (treatment 2), or at the end of the skewing process (treatment 3). In all three cases, the ligand was maintained in the cultures for a total of 7 days following its addition. The strongest inhibitory effects of 1,25(OH)$_2$D$_3$ on both IFN-γ and IL-4 production were observed when the hormone was present at the onset as well as throughout the differentiation process (Fig. 3, *B* and *E*). However, if polarization was allowed to proceed in the absence of

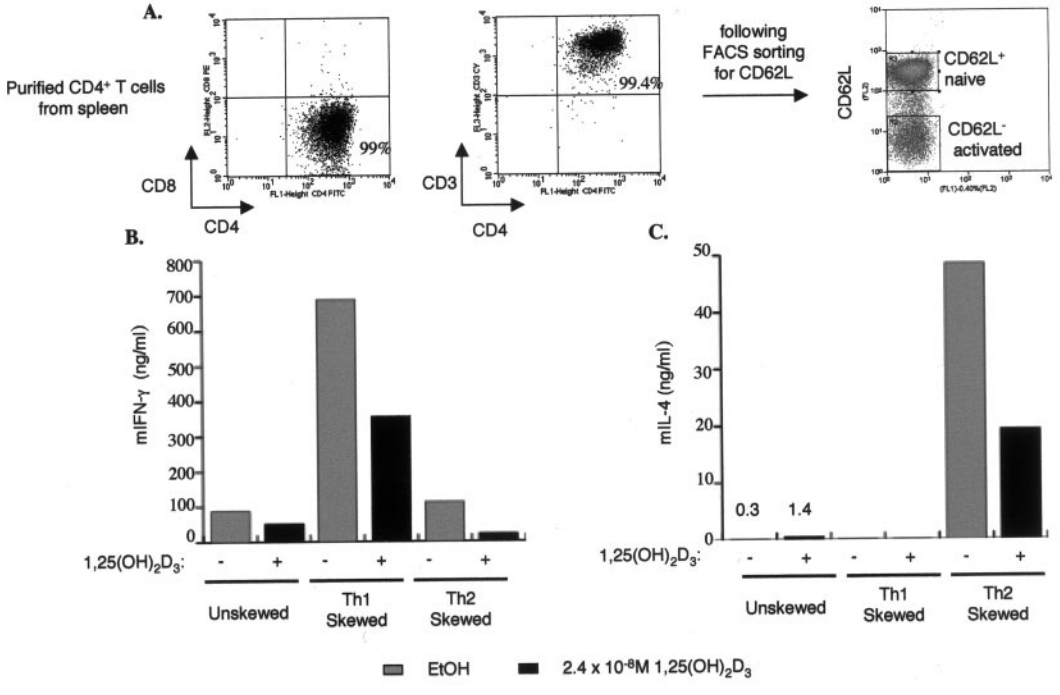

FIGURE 1. 1,25(OH)$_2$D$_3$ inhibits both Th1 and Th2 differentiation of naive primary murine CD4$^+$ T cells. *A*, Phenotype analysis of purified CD4$^+$CD62L$^+$ and CD62L$^-$ T cells. *B* and *C*, Naive (CD62L$^+$) CD4$^+$ T cells were activated in vitro under either nonpolarizing, Th1-polarizing, or Th2-polarizing conditions in the presence (filled bars) or absence (shaded bars) of 2.4 × 10^{-8} M 1,25(OH)$_2$D$_3$. Cytokine production was measured by ELISA 7 days after the start of differentiation. A total of nine experiments were conducted yielding similar results. One representative experiment is shown.

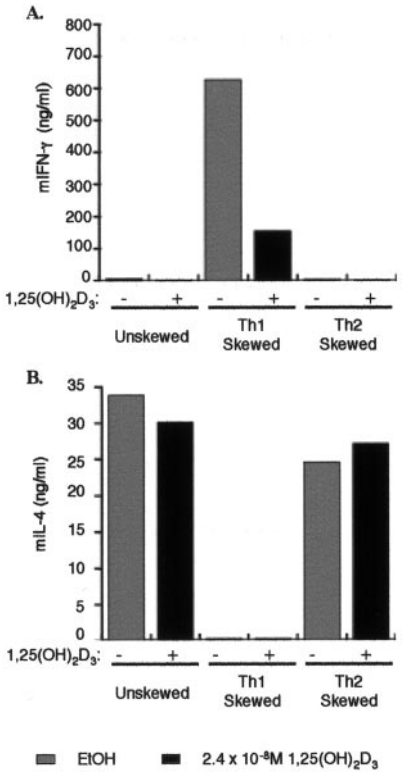

FIGURE 2. Effect of $1,25(OH)_2D_3$ on Th differentiation of activated $CD62L^-CD4^+$ T cells. $CD62L^-CD4^+$ T cells were stimulated in vitro under nonpolarizing, Th1-polarizing, or Th2-polarizing conditions in the presence (filled bars) or absence (shaded bars) of 2.4×10^{-8} M $1,25(OH)_2D_3$. Cytokine production was measured by ELISA 7 days after the start of differentiation. A representative experiment of 10 independent experiments with similar results is shown.

the ligand for as little as 4 days, the subsequent addition and maintenance of $1,25(OH)_2D_3$ in the cultures for 7 days was ineffective in diminishing the production of these two cytokines (Fig. 3, *C* and *F*). Similarly, addition of the hormone at the end of the differentiation process did not decrease the amount of secreted IFN-γ and IL-4 (Fig. 3, *D* and *G*). Thus, $1,25(OH)_2D_3$ is able to inhibit IFN-γ and IL-4 production in naive cells skewed toward the Th1 or Th2 subsets, respectively, only if present during the early stages of the polarization process.

Temporal effects of 1,25(OH)$_2$D$_3$ on CD62L$^-$CD4$^+$ T cells

Akin to the experiments just described, $CD62L^-$ T cells were differentiated in vitro in the presence of $1,25(OH)_2D_3$ added at the start of the polarization (treatment 1); halfway through the differentiation (treatment 2), or at the end of the skewing process (treatment 3) (Fig. 4*A*). In all three cases, the hormone was maintained in the cultures for a total of 7 days before the cells were washed and restimulated. As was the case with the naive cells, $1,25(OH)_2D_3$ suppressed IFN-γ production only when the hormone was provided at the onset of the differentiation process (Fig. 4*B*). Once the cells had been activated in vitro under strong skewing conditions for 4 days, the ligand was much less effective in inhibiting IFN-γ synthesis (Fig. 4*C*). If $1,25(OH)_2D_3$ was first added to the cells 7 days after the start of the in vitro differentiation, the ligand no longer inhibited (Fig. 4*D*). $1,25(OH)_2D_3$ had no effect on IL-4 production from $CD62L^-$ T cells during their in vitro differentiation (Fig. 4, *E–G*). The inhibitory effect of $1,25(OH)_2D_3$ on $CD62L^-$ T cells during their in vitro Th differentiation seems to be confined to IFN-γ only. Furthermore, this suppression is dependent on the presence of the ligand during the early stages of the polarization process.

1,25(OH)$_2$D$_3$ inhibits IFN-γ and IL-4 production from naive CD4$^+$ T cells in the absence of a cell cycle block

Having observed that $1,25(OH)_2D_3$ inhibits both IFN-γ and IL-4 production from naive $CD4^+$ T cells as well as IFN-γ synthesis from $CD62L^-CD4^+$ T cells during their in vitro polarization, we wanted to determine the mechanism of this repression. There are at least three possible ways that may account for inhibition of IFN-γ and IL-4 caused by $1,25(OH)_2D_3$ during the differentiation process: 1) a block in the cell cycle of the differentiating cells, 2) an inhibition of the expression of the Th1 and Th2 transcription factors, and 3) a transcriptional repression of the IFN-γ and IL-4 loci.

We were especially intrigued by the possibility that $1,25(OH)_2D_3$ may inhibit the cell cycle in differentiating Th cells, as previous work from our laboratory has shown that $1,25(OH)_2D_3$ inhibits the cell cycle by up-regulating p21 and p27 in the myelomonocytic cell line U937 (43). Furthermore, Rigby and colleagues (44) have shown that $1,25(OH)_2D_3$ blocks the cell cycle in human T cells. Considering that cell cycle progression provides an important level of regulation governing Th differentiation (45), we wondered whether $1,25(OH)_2D_3$ may be able to inhibit the key Th1 and Th2 cytokines, namely IFN-γ and IL-4, in naive cells by virtue of its ability to inhibit the cell cycle. To address this question, naive T cells were labeled with CFSE (Fig. 5*A*) and subsequently activated in vitro and differentiated as described for the experiments in Fig. 1. The cell division status of the cells was followed every 24 h by FACS. CFSE labels cells by spontaneously and irreversibly coupling to cellular proteins. In addition, it is distributed equally between daughter cells; thus, the CFSE intensity is halved with each cell division cycle (46). Importantly, CFSE does not interfere with the proliferative and differentiative capacity of the cells (45, 47). Treatment of naive T cells with $1,25(OH)_2D_3$ did not inhibit the cell cycle in any of the Th differentiation conditions (compare Fig. 5, *B* and *C*). Although only the day 3 data are shown, similar results were obtained at all other days tested (data not shown).

It has been shown that IL-2 can reverse the cell cycle block of $1,25(OH)_2D_3$ on human PBMC (48). Because our experiments were done in the presence of exogenous IL-2, it is formally possible that the inability to detect any changes in cell cycle progression could be due to the addition of IL-2 to the cultures. Thus, we have also performed the cell cycle analysis experiments in the absence of exogenous IL-2 and, as we observed with IL-2, we do not see any significant differences in the CFSE profiles of the $1,25(OH)_2D_3$-treated vs the untreated cells (data not shown). To confirm that $1,25(OH)_2D_3$ was able to inhibit IFN-γ and IL-4 production despite the lack of a cell cycle block, supernatants were collected 24 h after the start of the secondary stimulation and analyzed for these cytokines. As shown previously (Fig. 1, *B* and *C*), $1,25(OH)_2D_3$ strongly inhibited both IFN-γ (Fig. 5*D*) and IL-4 (Fig. 5*E*) production from the naive cells. Thus, $1,25(OH)_2D_3$ inhibits production of the signature Th1 and Th2 cytokines from naive T cells during their in vitro polarization by a mechanism independent of a cell cycle block.

1,25(OH)$_2$D$_3$ does not affect the cell cycle in CD62L$^-$CD4$^+$ cells during in vitro Th differentiation

Because $1,25(OH)_2D_3$ retained the capacity to inhibit IFN-γ production even in $CD62L^-$ T cells (Figs. 2*A* and 4*B*), provided the ligand was present from the beginning of the Th differentiation, we wanted to test whether $1,25(OH)_2D_3$ inhibits the cell cycle in these

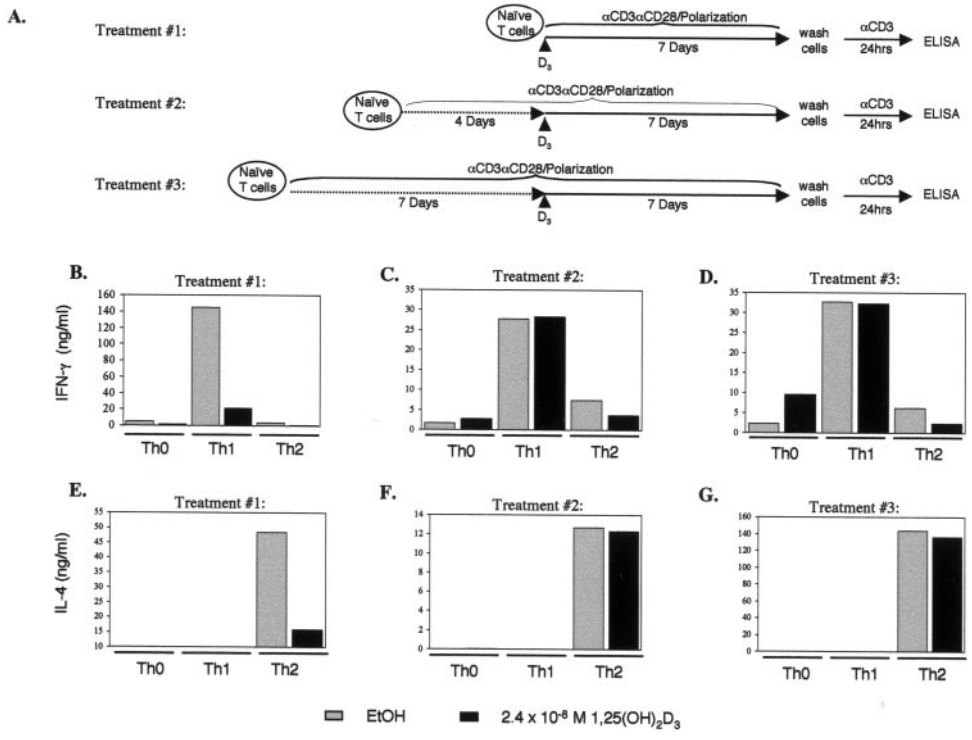

FIGURE 3. Inhibition of Th1 and Th2 differentiation of naive $CD4^+$ T cells by $1,25(OH)_2D_3$ is dependent upon the presence of this ligand during the early stages of the polarization process. *A*, Schematic representation of the experimental setup. Naive ($CD62L^+$) $CD4^+$ cells were stimulated with immobilized anti-CD3 and anti-CD28 Abs under Th1- or Th2-polarizing conditions for 7 days and were assayed for cytokine production following a 24-h stimulation with anti-CD3. $1,25(OH)_2D_3$ was added at the beginning of differentiation (day 0, *B* and *E*), 4 days following the start of differentiation (day 4, *C* and *F*), or at the end of the primary culture (day 7, *D* and *G*). It should be noted that regardless of when the hormone was first added to the primary cultures, $1,25(OH)_2D_3$ was subsequently maintained with the cells for a total of 7 days before the cultures were restimulated. The level of secreted murine IFN-γ (*B–D*) or murine IL-4 (*E–G*) was measured by ELISA. The nanogram per milliliter levels vary from graph to graph due to the different numbers of cells used during the secondary stimulation in each experiment. However, an equal number of cells were used per condition within each experiment.

cells during the polarization process. Thus, similar to the experiments described above, $CD62L^-CD4^+$ T cells were labeled with CFSE (Fig. 6*A*) and differentiated in vitro. The number of cell divisions was monitored every 24 h by FACS. Analogous to the lack of a cell cycle effect of $1,25(OH)_2D_3$ in naive cells, this hormone also did not inhibit the cell cycle of $CD62L^-$ cells (compare

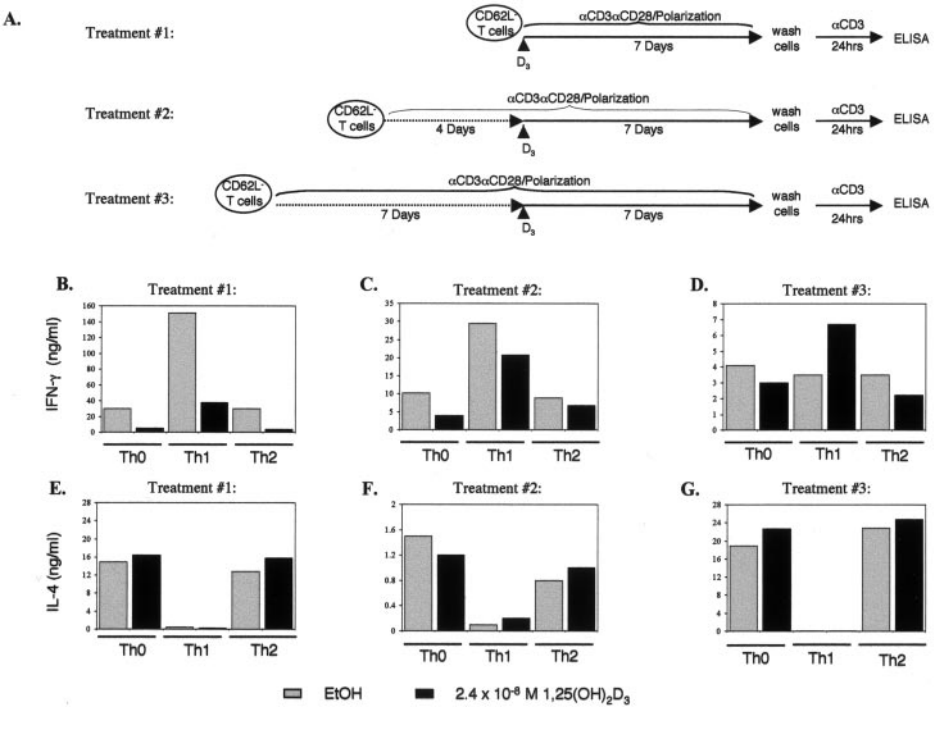

FIGURE 4. $1,25(OH)_2D_3$ inhibits Th1 but not Th2 differentiation in $CD62L^-CD4^+$ T cells. *A*, $CD62L^-CD4^+$ cells were stimulated with anti-CD3 and anti-CD28 under Th1- or Th2-polarizing conditions for 7 days and were assayed for cytokine production following a 24-h stimulation with anti-CD3. $1,25(OH)_2D_3$ was added at the start of differentiation (day 0, *B* and *E*), 4 days following the start of differentiation (day 4, *C* and *F*), or at the end of the primary culture (day 7, *D* and *G*). It should be noted that regardless of when the hormone was first added to the primary cultures, $1,25(OH)_2D_3$ was subsequently maintained with the cells, under the skewing conditions, for a total of 7 days before the cultures were restimulated. The level of secreted murine IFN-γ (*B–D*) or murine IL-4 (*E–G*) was measured by ELISA. The nanogram per milliliter levels vary from graph to graph due to the different numbers of cells used during the secondary stimulation in each experiment. However, an equal number of cells were used per condition within each experiment.

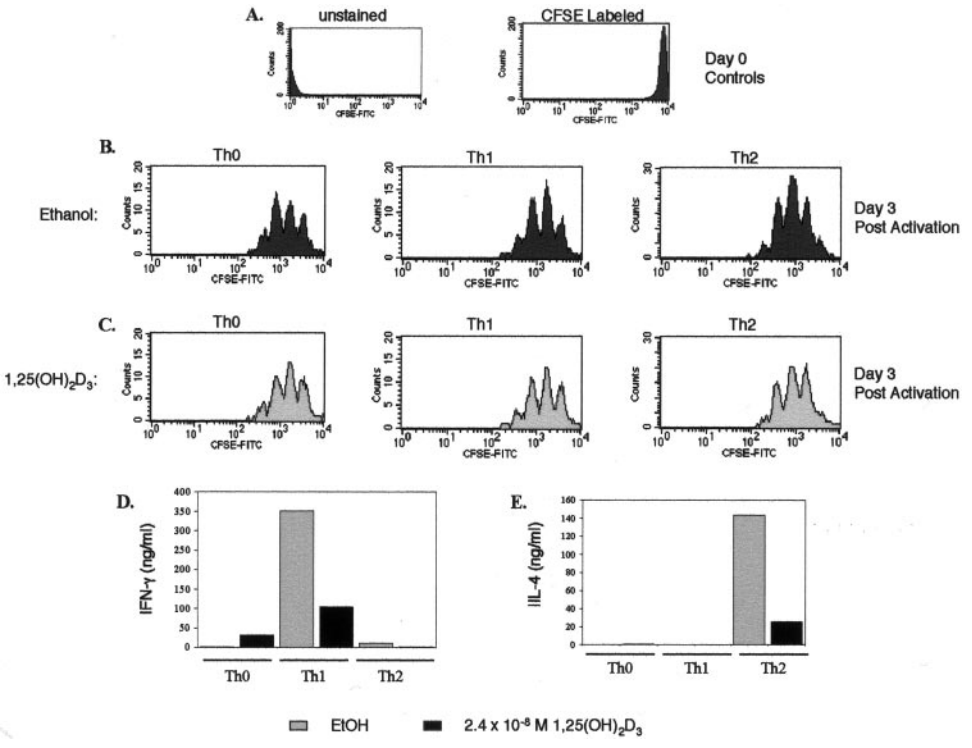

FIGURE 5. $1,25(OH)_2D_3$ inhibits the differentiation of naive $CD4^+$ T cells in the absence of a cell cycle block. *A*, Naive $CD4^+$ T cells were labeled with CFSE and differentiated in vitro as previously described. *B* and *C*, The cell division status of the differentiating cells was assayed by FACS every 24 h following activation. A representative time point, day 3, is shown. *D* and *E*, ELISAs on the culture supernatants at the end of the differentiation process confirmed the presence of Th1 and Th2 inhibition by liganded VDR despite the lack of a cell cycle block.

Fig. 6, *B* and *C*). Once again, the lack of a cell cycle block was correlated with the previously observed inhibition of IFN-γ (Fig. 6*D*) and no effect on IL-4 (Fig. 6*E*) production in $CD62L^-$ T cells. $1,25(OH)_2D_3$ treatment also did not affect the viability of the $CD62L^+$ or $CD62L^-$ cells as measured by trypan blue exclusion (data not shown). Thus, as is the case with naive cells, $1,25(OH)_2D_3$ inhibits IFN-γ in $CD62L^-$ T cells via a mechanism which does not involve a cell cycle block.

VDR does not alter the levels of the Th1 and Th2 transcription factors during the in vitro polarization of $CD4^+$ T cells

The effects of $1,25(OH)_2D_3$ on in vitro polarization of $CD4^+$ T cells could conceivably be mediated through the inhibition of key T cell transcription factors. To test whether $1,25(OH)_2D_3$ inhibits the Th1 transcription factor T-bet (49) or the Th2 transcription factors GATA-3 (50) or c-Maf (51), we isolated total RNA from in

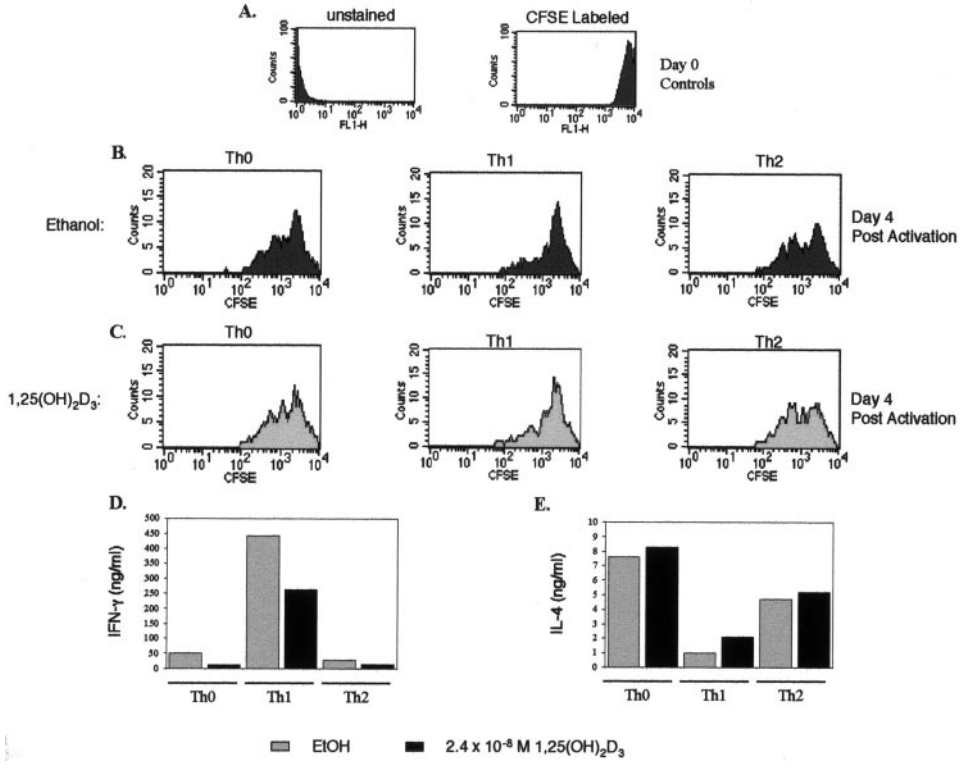

FIGURE 6. $1,25(OH)_2D_3$ does not affect the cell cycle in differentiating $CD62L^-CD4^+$ cells. *A*, $CD62L^-CD4^+$ cells were labeled with CFSE and differentiated in vitro. *B* and *C*, The cell division status of the differentiating cells grown in the presence (filled histograms) or absence (shaded histograms) was assayed by FACS. A representative time point, day 4, is shown. *D* and *E*, ELISAs on the culture supernatants from the secondary stimulation.

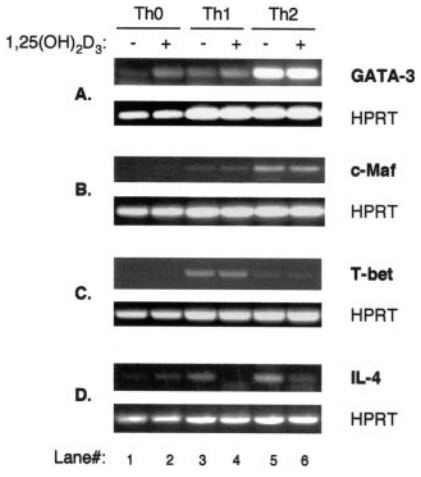

FIGURE 7. Effect of $1,25(OH)_2D_3$ on the Th1 and Th2 transcription factors and IL-4 mRNA levels. Total RNA obtained from activated and nonpolarized or polarized naive $CD4^+$ T cells was analyzed by RT-PCR for levels of the Th2 transcription factors GATA-3 (*A*) and c-Maf (*B*) as well as the Th1 transcription factor T-bet (*C*) and the Th2 cytokine IL-4 (*D*).

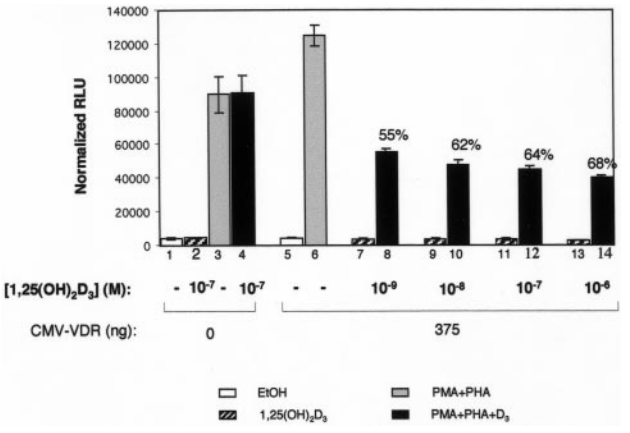

FIGURE 8. VDR inhibits activated transcription of the murine IL-4 promoter in the presence of $1,25(OH)_2D_3$. Jurkat T cells were transfected with an 800-bp IL-4 promoter reporter construct (54) as well as 375 ng of CMV-VDR DNA. Cells were treated for 8 h with the activating agents PHA and PMA (shaded bars) and/or increasing concentrations of $1,25(OH)_2D_3$ (filled bars). Luciferase activity was normalized to total protein as well as β-galactosidase activity produced off the internal control plasmid CMV-β-gal that was included in each transfection.

vitro activated naive $CD4^+$ T cells at the end of the polarization process and performed RT-PCR analysis. Our data show that the hormone does not alter the levels of GATA-3 (Fig. 7*A*, compare *lanes 5* and *6*) in cells polarized toward the Th2 condition. However, interestingly, $1,25(OH)_2D_3$ does enhance the levels of GATA-3 in nonpolarized cells (Fig. 7*A*, compare *lanes 1* and *2*). This correlates with the enhancement in IL-4 production observed under nonpolarizing conditions with the ELISA (Fig. 1*C*). Similarly, the expression of the other Th2-specific transcription factor, c-Maf, was not altered by the presence of the hormone (Fig. 7*B*, compare *lanes 5* and *6*). Likewise, $1,25(OH)_2D_3$ did not influence the expression of the Th1-specific transcription factor T-bet (Fig. 7*C*, compare *lanes 3* and *4*). We have also performed these assays in $CD62L^-CD4^+$ T cells and, similar to our results in naive cells, we find that there is no effect of $1,25(OH)_2D_3$ on the levels of the Th1 and Th2 transcription factors (data not shown). This correlates with the lack of an effect of this hormone on IFN-γ and IL-4 production as measured by ELISA (Fig. 4). To confirm the potency of our ligand, as well as to test the ability of the hormone to modulate IL-4 expression at the transcriptional level, we assayed the mRNA levels of IL-4 in the presence or absence of the ligand. $1,25(OH)_2D_3$ caused a significant down-regulation of IL-4 mRNA levels in polarized cells (Fig. 7*D*, compare *lanes 5* and *6*), suggesting that this hormone inhibits IL-4 production via transcriptional repression.

VDR inhibits transcription of the murine IL-4 promoter in a ligand dose-dependent manner

Previous studies from our laboratory have shown that $1,25(OH)_2D_3$ inhibits IL-2 and GM-CSF transcription through a mechanism involving interference or competition of VDR with NFAT/AP-1 DNA binding (29, 35, 38). Because both IL-4 protein and mRNA levels were inhibited in the presence of $1,25(OH)_2D_3$ (Figs. 1*C* and 7*D*), and because the murine IL-4 promoter contains five NFAT binding sites within the proximal 300 bp (reviewed in Refs. 52 and 53), we hypothesized that $1,25(OH)_2D_3$ may directly modulate the activity of this locus at the transcriptional level. To test this possibility, Jurkat cells were cotransfected by electroporation with a VDR producer plasmid (CMV-VDR), an IL-4 reporter plasmid containing 800 bp of the proximal murine promoter (54), and an internal control plasmid (CMV-β-galactosidase). The cells were treated with activating agents, PHA and PMA, in the presence (filled bars) or absence (shaded bars) of $1,25(OH)_2D_3$. In addition, ethanol (open bars) and $1,25(OH)_2D_3$ (hatched bars) treatment controls were included. Eight hours postactivation, the cells were harvested and luciferase levels were measured and normalized for protein concentration and β-galactosidase activity. In the absence of VDR, $1,25(OH)_2D_3$ treatment did not affect IL-4 promoter activity (Fig. 8, compare *lanes 3* and *4*). Overexpression of VDR resulted in a dose-dependent repression of activated transcription in the presence of 10^{-9}–10^{-6} M $1,25(OH)_2D_3$ (Fig. 8, compare *lanes 8*, *10*, *12*, and *14* to *lane 6*). Thus, inhibition of IL-4 transcription by ligand-bound VDR appears to be one mode by which $1,25(OH)_2D_3$ suppresses IL-4 production.

Discussion

We have examined the effect of $1,25(OH)_2D_3$ on the Th differentiation process in naive ($CD62L^+$) as well as activated ($CD62L^-$) $CD4^+$ T cells. Our results show that this hormone inhibits not only IFN-γ but also IL-4 production in naive cells differentiated toward the Th1 or Th2 subset, respectively. $1,25(OH)_2D_3$ maintained its ability to suppress IFN-γ synthesis even in $CD62L^-$ cells but was unable to attenuate IL-4 expression in these cells. Our experiments further revealed that $1,25(OH)_2D_3$ was needed during the early stages of the differentiation process to exert its inhibitory effect on IL-4 and IFN-γ production. If the cells were allowed to differentiate under strong polarizing conditions in the absence of this ligand for as few as 4 days, $1,25(OH)_2D_3$ was ineffective in suppressing either of the two cytokines.

As presented here, it is clear that $1,25(OH)_2D_3$ does not alter cell cycle progression in naive or in vivo activated $CD4^+$ cells differentiated into either subset in vitro. $1,25(OH)_2D_3$ also did not alter the levels of Th1/Th2 transcription factors, suggesting that the inhibition of IFN-γ and IL-4 is not secondary to the inhibition of these key transcription factors. We also found that $1,25(OH)_2D_3$ does not alter the levels of the IL-4 receptor (data not shown). Instead, our preliminary data show that VDR can directly down-regulate IL-4 transcription (Figs. 7*D* and 8), suggesting that this may be the basis for the diminished IL-4 levels in the presence of this liganded receptor. It is interesting to note the lack of an effect of $1,25(OH)_2D_3$ on IL-4 production from memory/activated

($CD62L^-$) $CD4^+$ T cells despite the fact that this hormone most likely inhibits IL-4 transcription. We believe that chromatin remodeling, which occurs during Th differentiation at the IL-4 locus, may prevent VDR from binding to cognate response elements, thus eliminating suppression of IL-4 production in memory/activated cells.

While our data clearly demonstrate a role for $1,25(OH)_2D_3$ in modulating the levels of the key Th1 and Th2 cytokines during in vitro polarization of naive $CD4^+$ T cells, the question still remains as to whether this hormone inhibits Th differentiation per se or whether it simply inhibits cytokine production from differentiating or already differentiated cells. The lack of an effect of $1,25(OH)_2D_3$ on both the cell cycle and the key Th1/Th2 transcription factors during the polarization of $CD4^+$ T cells would argue against a general block on Th differentiation by this ligand. Instead, our data are more consistent with a model in which $1,25(OH)_2D_3$ inhibits cytokine production during $CD4^+$ T cell differentiation via transcriptional repression of IL-4 in the case of Th2-polarized cells. Similarly, Cippitelli and Santoni (36) have demonstrated the down-modulation of IFN-γ transcription by $1,25(OH)_2D_3$. Thus, a common strategy in which $1,25(OH)_2D_3$ may hamper the function of Th1 and Th2 cells is through the transcriptional down-regulation of central cytokines such as IFN-γ and IL-4.

To the best of our knowledge, this is the first study describing the effects of $1,25(OH)_2D_3$ on purified naive or $CD62L^-CD4^+$ T cells during their in vitro polarization. Lemire and colleagues (40) used human rye grass allergen-specific T cell clones, isolated from atopic patients and classified into Th0, Th1, or Th2 subsets based on their cytokine secretion profile, and activated them in vitro with HLA-matched APCs in the presence or absence of $1,25(OH)_2D_3$. Forty-eight to 72 h postactivation, the supernatants revealed decreased levels of IFN-γ but unaltered levels of IL-4 (40). These data are in accord with our observations on the effect of $1,25(OH)_2D_3$ on $CD62L^-$ murine $CD4^+$ T cells, where this hormone also inhibits IFN-γ production (Fig. 2*A*) but does not affect IL-4 production (Fig. 2*B*).

Earlier studies by Lemire and Archer (55) showed that $1,25(OH)_2D_3$ prolonged the survival of mice immunized with "encephalitogenic doses of central nervous tissue"; in other words, $1,25(OH)_2D_3$ suppressed murine experimental autoimmune encephalomyelitis (EAE). In addition, they also demonstrated that this hormone suppressed anti-MBP Ab production. Based on their results, they suggested that the hormone may inhibit in vivo T cells central to the development of EAE. They also argued that the significant recovery detected in $1,25(OH)_2D_3$-treated mice despite severe clinical disease may be due to the inability of the mice to develop an Ab response. The fact that $1,25(OH)_2D_3$ was able to inhibit Ab production in this model coupled to the well-established role of IL-4 in aiding Ab production by B cells lends indirect support to our findings of suppression of IL-4 by this hormone.

Following the initial demonstration of the beneficial effects of $1,25(OH)_2D_3$ on EAE, Cantorna and colleagues (56) extended those findings by reporting that this hormone reversibly blocks the progression of EAE while vitamin D_3 deficiency accelerates the onset of the disease. A subsequent study by the same group argued that $1,25(OH)_2D_3$ treatment of mice with EAE caused an increase in IL-4 and TGF-β1 mRNA levels in the lymph nodes, spinal cord, and brain (57). Based on these observations, they suggested that $1,25(OH)_2D_3$ is a positive regulator of these two cytokines and that this may account for the ability of this hormone to block encephalomyelitis (57). Our results demonstrating that $1,25(OH)_2D_3$ diminishes IL-4 production from naive $CD4^+$ T cells challenge these observations. However, several important differences exist between the experimental systems used in the two studies, which may perhaps explain the disparity in results, including the strains of mice, the time course of the experiments, the purity of the cell populations, and the difference in the type of activation stimuli used.

The exact role of $1,25(OH)_2D_3$ on Th2 differentiation remains unclear. Our results may suggest an inhibitory role for this hormone on Th2 function and perhaps differentiation while an earlier report suggests only minimal effects on Th2 development (58). Recently, an enhanced Th2 differentiation by $1,25(OH)_2D_3$ was suggested (59). While we have concentrated our studies on the inhibitory effects of $1,25(OH)_2D_3$ on IFN-γ and IL-4 during in vitro polarization of $CD4^+$ T cells, it should be noted that under nonskewing conditions (i.e., the Th0 condition) we see a modest enhancement of IL-4 production (Fig. 1*C*) as well as an increase in the Th2 transcription factor GATA-3 (Fig. 7*A*, *lanes 1* and *2*). This is in accord with the recent report by Boonstra and colleagues (59), who show that $1,25(OH)_2D_3$ enhances IL-4 production and GATA-3 levels following in vitro activation of $CD4^+$ T cells under nonskewing conditions. However, unlike the reported increase in c-Maf mRNA (59), we do not detect any appreciable levels of this transcription factor in our nonpolarized cultures. The ability of $1,25(OH)_2D_3$ to inhibit IL-4 levels under polarizing conditions but to enhance levels of this cytokine under nonpolarizing conditions suggests a differential regulation of IL-4 by this hormone. This modulation may be dependent on the cytokine environment.

The data presented in this study establish the autonomous effects of $1,25(OH)_2D_3$ on the functional potential of Th cells. We have demonstrated the importance of the presence of this hormone during the early stages of the differentiation process to ensure its effectiveness in suppressing both IFN-γ and IL-4 synthesis in naive cells polarized toward the Th1 or Th2 condition, respectively. There are two possible implications of this effect of $1,25(OH)_2D_3$ on Th differentiation. On one hand, the requirement for an early involvement of this hormone in the Th polarization process may suggest that $1,25(OH)_2D_3$ interferes with the establishment of the Th1/Th2 subsets. However, the lack of an effect of $1,25(OH)_2D_3$ on the cell cycle and the Th1/Th2 transcription factors argues against that possibility. Instead, the fact that $1,25(OH)_2D_3$ suppresses IFN-γ and IL-4 production may suggest that the ligand plays a role in the maintenance of the Th subsets. This notion may also be supported by the absence of a complete block in Th differentiation by $1,25(OH)_2D_3$. Additional experiments will be needed to discern whether $1,25(OH)_2D_3$ interferes with the establishment or the maintenance of the Th1 and Th2 subsets. At present, the ability of $1,25(OH)_2D_3$ to inhibit key Th cytokines during in vitro differentiation suggests that this hormone may likely hinder the function of differentiating Th cells.

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