Interactions of 1α,25-dihydroxyvitamin D3 with IL-12 and IL-4 on cytokine expression of human T lymphocytes

Ralf Thien, Karin Baier, Peter Pietschmann, MD, Meinrad Peterlik, PhD, MD, and Martin Willheim, MD Vienna, Austria

Background: 1α,25-Dihydroxyvitamin D3 (1α,25(OH)2D3) exerts its effects on the immune system, particularly through suppression of T helper/cytotoxic cell 1 (Th1/TC1)-mediated reactions, although direct actions of 1α,25(OH)2D3 on human T lymphocytes have not yet been studied in detail.

Objective: We evaluated the effect of 1α,25(OH)2D3 on basal and cytokine-driven T-cell functions at the single-cell level.

Methods: We used 4-color flow cytometry for simultaneous detection of intracellular cytokines in CD4+ and CD8+ human PBMCs that had been cultured in the presence of 1α,25(OH)2D3 singly or in combination with either IL-12 or IL-4. According to the exploratory nature of these investigations, the Bonferroni correction was not applied for data analysis and presentation.

Results: 1α,25(OH)2D3 had little effect on Th1/TC1 cytokines but significantly inhibited IL-12–induced IFN-γ production. Constitutive synthesis of Th2/TC2-related cytokines was also only modestly affected by 1α,25(OH)2D3 alone. When Th0/Th2 differentiation was induced by IL-4, 1α,25(OH)2D3 significantly reduced the percentages of IL-4+ and IL-13+ cells. However, the predominant effect of 1α,25(OH)2D3 on T lymphocytes, particularly in the presence of IL-4, was the induction of separate CD4+ and CD8+ subpopulations with almost exclusive expression of IL-6. This might be an important facet of the immunomodulatory action of 1α,25(OH)2D3 because IL-6 might act in parallel with 1α,25(OH)2D3 in modulation of TTh1/Th2 effector cell functions.

Conclusions: Our data imply that the specific actions of 1α,25(OH)2D3 on cytokine-stimulated T-cell functions could play a role in the prevention of Th2/Th1-related autoimmune diseases but also predispose toward Th0/Th2-mediated allergic reactions. (J Allergy Clin Immunol 2005;116:683-9.)

Key words: Vitamin D, immune system, CD4, CD8, IL-2, IL-6, IL-13, IFN-γ, allergy, autoimmune disease

Teff lymphocyte responses are mediated by 2 functionally distinct CD4+ T-cell subsets, which can be distinguish on the basis of their different cytokine production profiles.1,4 Th1 cells produce IFN-γ and IL-2 but little or no IL-4 and IL-5, respectively, whereas Th2 cells mainly produce IL-4, IL-5, and also IL-13. Corresponding cytokine production patterns were found also in 2 subsets of the CD8+ T-lymphocyte population (ie, in Tc1 and Tc2 cells).4,6

The local cytokine environment plays an important role in the differentiation of T cells along the Th1/Th2 pathway.3,4 IL-4 is one of the most potent inducers of Th2 development,7,10 whereas IL-12, which is secreted mainly by antigen-presenting cells, initiates differentiation of Th1 cells9,10 and downregulates Th2 cell expansion. The differentiation of CD8+ lymphocytes into Tc1- and Tc2-type cells is triggered by the same agents that promote Th1 and Th2 development.11,12

The steroid hormone 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) is the biologically active metabolite of vitamin D, which plays an important role in mineral homeostasis but has also been identified as a potent modulator of immune responses.13-18 There is evidence that in animals 1α,25(OH)2D3 prevents the development of experimental autoimmune diseases, such as insulin-dependent diabetes mellitus, allergic encephalomyelitis, autoimmune thyroiditis, and inflammatory bowel disease.19-24 Epidemiologic studies suggest that in human subjects high vitamin D intake reduces the risk, for example, for type I diabetes mellitus and rheumatoid arthritis.25,26 It must be noted, however, that vitamin D, when given in early childhood, not only prevents rickets and juvenile diabetes25 but at the same time seems to increase the risk of allergic diseases in later life.27

The preventive effect on autoimmune diseases can be explained by findings that 1α,25(OH)2D3, when added to cultures of murine or human PBMCs, suppresses the release of typical Th1-type cytokines (ie, IL-2, IFN-γ, or TNF-α).19,26-32 It is assumed that as a consequence of suppression of Th11 cytokines by 1α,25(OH)2D3, the prevalence of Th12-type immune reactions creates a condition that certainly favors the development of allergic diseases. However, it is unclear in which way 1α,25(OH)2D3...
modulates human T$_{H}2$ cell function because until now, respective studies were done only with murine PBMCs and, in addition, yielded rather conflicting results.

To obtain more detailed information on the direct effects of 1$\alpha$,25(OH)$_2$D$_3$ on the differentiation and function of individual T-lymphocyte subpopulations, we developed a protocol for evaluation of cytokine production at the single-cell level. Using 4-color flow cytometric analysis, we were able to assess the intracellular content of 2 different cytokines simultaneously in both the CD4$^+$ and CD8$^+$ populations of cultured human PBMCs. In a previous study we could show that 1$\alpha$,25(OH)$_2$D$_3$ induced a small but consistent reduction of T cells producing IL-2 and increased the percentage of T cells positive for IL-4 and IL-13. Notably, 1$\alpha$,25(OH)$_2$D$_3$ caused the appearance of a substantial T-cell subpopulation, which produced mainly IL-6 and could not be classified as T$_H$/T$_C1$ or as T$_H$/T$_C2$. In the present study we assessed, for the first time, the interaction of 1$\alpha$,25(OH)$_2$D$_3$ with IL-12 or IL-4, respectively, on coexpression profiles of IL-2, IFN-$\gamma$, IL-4, IL-13, and IL-6 in CD4$^+$ and CD8$^+$ cells to get deeper insight into the cooperative effects of 1$\alpha$,25(OH)$_2$D$_3$ with T$_H$/T$_C1$- and T$_H$/T$_C2$-differentiating cytokines on specific effector functions of various T-lymphocyte subsets.

**METHODS**

**Culture conditions**

Human PBMCs were isolated from the heparinized blood of 6 healthy donors (3 male and 3 female subjects; age, 23-36 years) by means of Ficoll-Paque density gradient centrifugation. PBMCs were seeded at a density of 10$^6$/mL and cultured for up to 21 days in Ultra Culture Medium (Bio Whittaker, Walkersville, Md) supplemented with 2 mM L-glutamine (Sigma Bio Sciences, St Louis, Mo) and 170 mg/L gentamicin sulfate (Sigma Bio Sciences).

Cells from the same donor were treated in different ways (addition of different substances) under otherwise absolutely identical experimental conditions. For the first 3 days, aliquots of a PHA solution of different substances were added to achieve a concentration of 1% vol/vol. Thereafter, IL-2 (20 U/mL; Roche Diagnostics GmbH, Basle, Switzerland) was added to PBMC cultures to maintain cell proliferation and viability. Cultures were exposed to 1$\alpha$,25(OH)$_2$D$_3$ (10$^{-8}$ M; a kind gift from Hoffmann La-Roche, Basle, Switzerland), IL-4 (500 U/mL; Genzyme, Boston, Mass), or IL-12 (200 U/mL; a generous gift from M. Gately, Hoffmann La-Roche, Nutley, NJ), as indicated. On days 7 and 14, cells were washed once, and fresh medium and treatments were added.

**Intracellular cytokine detection**

Expression of IFN-$\gamma$, IL-2, IL-4, IL-6, and IL-13 in the T-lymphocyte fraction of PBMCs was assessed by using the previously described 4-color flow cytometric technique. On days 7, 14, or 21, cells were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate and 1.25 $\mu$M ionomycin in the presence of 2.0 $\mu$M monensin (all from Sigma Bio Sciences). After 4 hours, cells were harvested, washed, and fixed with 2% formaldehyde.

Four-color fluorescence staining was performed with rat or mouse anti-human mAb and the respective isotype controls labeled with FITC, phycoerythrin (PE), peridinin chlorophyll protein, or allophy-cocyanin. Anti-CD4 (allophycocyanin) and anti-CD8 (peridinin chlorophyll protein) were purchased from Becton Dickinson (San Jose, Calif), and anti-IFN-γ-FITC, anti-IL-2 (PE), anti-IL-4 (PE), anti-IL-6 (PE), and anti-IL-13 (PE) were obtained from Pharmingen (San Diego, Calif).

For the study of coexpression of IL-6 with IL-2, IL-4, and IL-13, a FITC-labeled anti-IL-6 mAb (Pharmingen) was used. In this case a polyconal FITC-labeled rabbit anti-rat IgG conjugate (STAR17B; Serotec, Oxford, United Kingdom) had to be used as a second-step reagent to reach sufficient signal intensity for IL-6. Percentages of IL-6$^+$ cells after optimal staining were similar with FITC and PE. Double-staining controls confirmed that the population detected by using both techniques was identical.

The gating strategy used to analyze cytokine (co)expression in CD4$^+$ and CD8$^+$ lymphocytes is illustrated in Fig 1. Cells were gated as lymphocytes by their light scatter characteristics and subsequently defined as CD4$^+$ and CD8$^+$. Cells fulfilling both criteria (lymphocytes and CD4 or CD8, respectively) were further analyzed for their cytokine production pattern.

**Statistics**

Data were analyzed with the 2-tailed paired Student t test. All groups showed a normal distribution according to the Kolmogorow-Smirnov test. Significance of difference was assumed at a $P$ value of less than .05. According to the exploratory nature of these investigations, the Bonferroni correction was not applied for data analysis and presentation.

**RESULTS**

**Effect of 1$\alpha$,25(OH)$_2$D$_3$ on basal and IL-12-inducible T$_H$/T$_C1$ differentiation**

When PBMCs were cultured in the presence of 10$^{-8}$ M 1$\alpha$,25(OH)$_2$D$_3$, we, in accordance with our previously published data, observed a small but significant decrease in the percentage of IL-2$^+$ cells within the CD4$^+$ population at any time point tested (Fig 2). In CD8$^+$ lymphocytes significant inhibition of IL-2 production was only detected on day 14. An effect of 1$\alpha$,25(OH)$_2$D$_3$ on IFN-$\gamma$ production was detectable neither in CD4$^+$ nor in CD8$^+$ cells (Fig 2).

Incubation of PBMCs with IL-12 resulted in a consistent increase of IL-2$^+$ cells and an even more pronounced increase of IFN-$\gamma^+$ cells within both the CD4$^+$ and the CD8$^+$ T-cell subsets (Fig 2). Simultaneous addition of 1$\alpha$,25(OH)$_2$D$_3$ abolished the positive effect of IL-12 on the number of IL-2-producing CD4$^+$ T cells. Notably, 1$\alpha$,25(OH)$_2$D$_3$ completely blocked the IL-12–related increase in the percentage of IFN-$\gamma^+$-positive lymphocytes within the CD4$^+$ and CD8$^+$ subset on day 7. Thereafter, 1$\alpha$,25(OH)$_2$D$_3$–mediated inhibition gradually ceased toward the end of the culture period, although in CD8$^+$ cells a 50% reduction of IL-12–induced IFN-$\gamma$ production was still noticed on day 21 (Fig 2).

**Induction of T$_H$/T$_C2$-related cytokine production by 1$\alpha$,25(OH)$_2$D$_3$ and IL-4**

When cultures were treated with 1$\alpha$,25(OH)$_2$D$_3$ alone, significance was only reached for the IL-13$^+$ CD4$^+$ population on day 21 (Fig 3).
Cultures treated with IL-4 alone showed significantly increased percentages of IL-4– and IL-13–producing cells within the CD4^+ population. Similar tendencies among the CD8^+ population commonly did not reach significance, except for IL-4–producing CD8^+ cells on day 21.

Simultaneous addition of 1α,25(OH)2D3 and IL-4 dramatically increased the percentages of IL-4– and IL-13–producing CD4^+ and CD8^+ cells, so that on day 21, more than 4 times as many T lymphocytes as in control cultures stained positively for IL-4 or IL-13, respectively (Fig 3, A).

Induction of an IL-6^+ T-cell subpopulation by 1α,25(OH)2D3 and IL-4

We observed another cooperative effect between 1α,25(OH)2D3 and IL-4 when we studied the induction of IL-6 production in CD4^+ and CD8^+ T lymphocytes (Fig 3, B). Although in control cultures both T-cell subsets remained virtually negative for IL-6, 1α,25(OH)2D3 induced an IL-6–producing population that steadily increased over time to 8.3% in CD4^+ lymphocytes on day 21. No significant increase was detectable in CD8^+ cells. IL-6^+ cells were rarely present in cultures treated with IL-4 alone, although a slight increase could be noticed in CD4^+ and CD8^+ subsets on day 7 (Fig 3, B).

However, the combination of 1α,25(OH)2D3 and IL-4 remarkably augmented the number of IL-6–producing cells (Fig 3, B). In both CD4^+ and CD8^+ T lymphocytes, IL-4 caused a 5-fold amplification of the percentage of IL-6^+ cells induced by 1α,25(OH)2D3 alone (Fig 3, B).

IL-12 neither induced IL-6 production in T lymphocytes nor modulated the effect of 1α,25(OH)2D3 thereon (data not shown).

Coexpression of IL-6 with IL-4 or IL-13

Because of the pronounced effect of 1α,25(OH)2D3 and IL-4 on T-lymphocyte differentiation in the Th/Th2 direction (Fig 3, A), we analyzed the effect of the steroid hormone and the cytokine on coexpression of IL-6 with each of the 2 Th/Th2-type cytokines, IL-4 and IL-13 (Fig 4).

The higher number of IL-6–producing cells in cultures treated with 1α,25(OH)2D3 and IL-4 was due to the expansion of IL-6 single-positive (ie, IL-6^+/IL-4^-/IL-13^-), as well as coexpressing (ie, IL-6^-/IL-4^+/IL-13^-), cells, respectively. In contrast, IL-4 and IL-13 single-positive (ie, IL-6^-/IL-4^-/IL-13^-) cells were only modestly expanded by the combination of 1α,25(OH)2D3 and IL-4 compared with 1α,25(OH)2D3 alone (Fig 4). Similar results were obtained for CD8^+ lymphocytes (data not shown).

1α,25(OH)2D3 specifically modulates cytokine (co)expression of CD4^+ T lymphocytes

For further characterization of the 1α,25(OH)2D3 effect on the different pathways of cytokine-triggered Th/Th2 cell differentiation, we analyzed cytokine coexpression patterns in CD4^+ and CD8^+ PBMCs cultured for 21 days.
IL-12 generated a typical Th1/TC1 pattern inasmuch as a substantial fraction of CD4+ cells were single positive for IFN-γ (Fig E1, A, lower right quadrants). A distinct percentage of IFN-γ–producing cells stained positively also for IL-4 or IL-13, respectively (Fig E1, A, upper right quadrants), whereas coexpression of IFN-γ with IL-6 was negligible.

Addition of 1α,25(OH)2D3 to IL-12–treated PBMC cultures reduced IL-12–induced IFN-γ expression. IL-6+ cells, which emerged under the influence of 1α,25(OH)2D3, coexpressed IFN-γ, although only to a minor extent (Fig E1, A).

IL-4 induced a typical Th2/TC2 phenotype characterized by a significant proportion of cells producing only IL-4 and IL-13 but not IFN-γ. In IL-4–treated cultures the number of IL-6+ cells was low (Fig E1, A, upper left quadrants).

When PBMCs were cultured in the presence of IL-4 and 1α,25(OH)2D3, approximately one third of CD4+ T cells exhibited a remarkable phenotype inasmuch as they showed positive immunostaining only for IL-6 (Fig E1, A, upper left quadrants). IL-6/IFN-γ–coexpressing cells only represented a small proportion of all IL-6–producing cells.

Addition of 1α,25(OH)2D3 expanded the populations of IL-4-induced single-positive (ie, IL-4+/IFN-γ- or IL-13+/IFN-γ-) cells (Fig E1, A, upper right quadrants). The percentage of the IFN-γ single-positive cells and of those coexpressing IL-4 or IL-13 in IL-4–treated PBMC cultures was not changed by coculture with 1α,25(OH)2D3 (Fig E1, A).

1α,25(OH)2D3 specifically modulates cytokine (co)expression of CD8+ T lymphocytes

In IL-12–treated PBMC cultures, the proportion of IFN-γ–positive cells was higher in the CD8+ subset (see Fig E1, B, in the Online Repository in the online version of this article at www.mosby.com/jaci) than in the CD4+ subset (Fig E1, A). Conversely, IL-4 seemed to induce lower percentages of CD8+ cells producing IL-4 and IL-13 than of CD4+ cells (Fig E1). Nevertheless, the regulatory effects of 1α,25(OH)2D3 on cytokine coexpression patterns were similar to those observed in CD4+ cells. Again, in PBMC cultures treated with 1α,25(OH)2D3 plus IL-4, IL-6–producing cells were predominant, although less frequent than in the CD4+ population (Fig E1).
Mean percentages of IFN-γ single-positive CD8+ lymphocytes were higher in PBMC cultures treated with IL-4 plus 1α,25(OH)2D3 when compared with those treated with IL-4 alone (Fig E1, B). However, this effect of 1α,25(OH)2D3, which was not observed in CD4+ T lymphocytes (Fig E1, A), did not reach statistical significance. Remarkably, even when compared with control cultures, no decrease in the frequency of IFN-γ–producing T cells could be detected in cultures with simultaneous addition of IL-4 and 1α,25(OH)2D3 (data not shown).

**DISCUSSION**

Until now, it was assumed that the Th1 subset of CD4+ T cells is the classical target cell population for the immunomodulating action of 1α,25(OH)2D3, whereas only isolated effects of the hormone on CD8+ lymphocytes had been reported.28,29 We think it important to note that, consistent with previous reports from our laboratory,36,37 the present study also clearly shows that 1α,25(OH)2D3 regulates cytokine production in CD4+ and CD8+ T cells in parallel. Statistical analyses were performed according to the exploratory nature of these investigations. However, even more stringent analysis with the Bonferroni correction, although reducing a number of the significant changes, did not negate any of the key results described.

The immunomodulating effect of 1α,25(OH)2D3 is commonly attributed to its ability to suppress Th1-associated cytokine production.13,20,28,38 From the present study, it seems that constitutive production of Th1 cytokines is only marginally influenced by 1α,25(OH)2D3 because it caused only a small reduction in the percentage of IL-2–producing, mainly CD4+ cells and induced no consistent change in the IFN-γ–positive T-cell fraction. However, when Th1 cytokines were induced by IL-12, the inhibitory effect of 1α,25(OH)2D3 was more visible on IFN-γ than on IL-2 production.

It has been suggested that suppression of IFN-γ production by 1α,25(OH)2D3 results from inhibition of IL-12 secretion from costimulatory cells, such as monocytes, dendritic cells, or B cells.18,39 Results from our experiments in the present study clearly indicate that 1α,25(OH)2D3 also directly interferes with IL-12 action on CD4+ and CD8+ T cells. This is a novel facet of the suppressive action on Th1/Th17 lymphocyte differentiation and function, by which 1α,25(OH)2D3 prevents the development of Th1-mediated autoimmune diseases in...
experimental animals and most likely also in human subjects. Until now, it has been assumed that 1α,25(OH)₂D₃ interacts specifically with TH₁/Tc₁ cell function and has no effect on TH₂/Tc₂ cells. In the present study we confirmed previous data, showing that the effects of 1α,25(OH)₂D₃ on constitutive TH₂/Tc₂-related cytokine production in fact were rather modest (Fig 3). However, when T-cell differentiation into the TH₂/Tc₂ direction was promoted by addition of IL-4 to PBMC cultures, 1α,25(OH)₂D₃ provided a strong costimulatory signal for the induction of IL-4 and IL-13 production by T lymphocytes (Fig 3). Analysis of cytokine coexpression patterns revealed that 1α,25(OH)₂D₃ in combination with IL-4 expanded the populations of IL-4 and IL-13 single-positive/IFN-γ⁻ T cells (Fig E1 in the Online Repository in the online version of this article at www.mosby.com/jaci). No decrease in the frequency of IFN-γ-producing T cells could be detected in cultures with simultaneous addition of IL-4 and 1α,25(OH)₂D₃. Our data therefore strongly suggest that the prevalence of TH₂/Tc₂ over TH₁/Tc₁ cell functions in the presence of 1α,25(OH)₂D₃ is not only an indirect consequence from TH₂/Tc₁ suppression but also due to a stimulatory effect of 1α,25(OH)₂D₃ on IL-4-driven TH₂/Tc₂ differentiation. It must be noted that expansion of both IL-4⁺ and IL-13⁺ cells is accompanied by coexpression of IL-6, a phenotype not detectable in TH₁/Tc₂ cells induced by IL-4 alone (Fig E1). It is also evident from Fig 4 that 1α,25(OH)₂D₃ plus IL-4 treatment induced no change in the percentages of IL-4⁺/IL-6⁻ or, respectively, IL-13⁺/IL-6⁻ T cells. It should be emphasized that induction of a T-cell subpopulation with predominant expression of IL-6 (Figs 4 and E1) is the major effect of 1α,25(OH)₂D₃ on T lymphocytes. IL-4, which has negligible effects on IL-6 production by T lymphocytes (Fig 3), amplifies the effect of 1α,25(OH)₂D₃, so that between 30% and 40% of CD4⁺ T cells in long-term PBMC cultures eventually become IL-6⁺ (Fig 3, A).

We therefore wanted to know whether this was due to an independent effect of 1α,25(OH)₂D₃ or whether TH₂/Tc₂ cells under the influence of the hormone acquire the ability to produce IL-6. Results from flow cytometric analysis of cytokine coexpression patterns in CD4⁺ and CD8⁺ T lymphocytes (Figs 4 and E1) indicate that there is some overlap of IL-6 and TH₂/Tc₂-related cytokine expression. However, the majority of IL-6⁺ T cells stained negative for typical TH₂/Tc₂ cytokines (ie, IL-4 and IL-13). Also, coexpression of IL-6 and TH₂/Tc₁-related cytokines was rarely observed.

Previous studies in the murine system have led to conflicting results regarding effects of 1α,25(OH)₂D₃ on naive T cells. 33,34 In a recent study we have shown that 1α,25(OH)₂D₃ is able to suppress IL-12-triggered TH₂/Tc₁ differentiation, as well as IL-4-triggered TH₂/Tc₂ differentiation, of naive T cells from human cord blood, whereas it induces, particularly in the presence of IL-4, a similar IL-6-producing phenotype as in cells from adult donors. This indicates that 1α,25(OH)₂D₃ exerts substantially different effects on immature and naive T cells and on a more mature T-cell population in the peripheral blood of adults that consists, to a significant extent, of cells with a memory phenotype.

Taken together, our data provide strong evidence that 1α,25(OH)₂D₃ causes the appearance of a novel T-cell subpopulation, which typically produces IL-6 only and thus does not fit into the TH₁/Tc₁–TH₂/Tc₂ dichotomy. With respect to the latter, Diehl and Rincon have summarized the evidence that IL-6 also regulates TH₁ functions in a dual way: IL-6 inhibits TH₁ differentiation through an indirect effect on IFN-γ signaling. At the same time, IL-6 augments TH₂ differentiation by upregulation of IL-4 mRNA expression in T lymphocytes. Therefore we hypothesize that induction of an IL-6-producing T-lymphocyte subpopulation might be an important facet of the immunomodulating role of 1α,25(OH)₂D₃ because IL-6 could enhance the 2 key actions of 1α,25(OH)₂D₃ on TH₂/Tc effector cells (ie, suppression of basal and IL-12–induced TH₂/Tc₁ functions, as well as stimulation of IL-4-dependent TH₂/Tc₂ differentiation).

Predominance of TH₂/Tc₂ over TH₁/Tc₁ functions induced by 1α,25(OH)₂D₃ has been observed recently also in vivo. Matheu et al reported that in a murine model of pulmonary eosinophilic inflammation, vitamin D treatment augmented allergen-induced T-cell proliferation along with TH₂ cytokine (IL-4 and IL-13) and IgE production. Thus it seems most likely that active expansion of TH₂/Tc₂ effector cell populations by 1α,25(OH)₂D₃, as observed in the present study, is underlying the observation made in epidemiologic studies, namely that vitamin D prophylaxis predisposes toward development of allergic diseases in later life.

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REFERENCES


