$1\alpha,25(\text{OH})_2\text{D}_3$ Inhibits Not Only Th1 But Also Th2 Differentiation in Human Cord Blood T Cells

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ABSTRACT

Human naive CD4^+ T helper (Th) and CD8^+ cytotoxic (Tc) T cells, which only produce IL-2, may differentiate into Th1/Tc1- or Th2/Tc2-like lymphocytes, characterized by their cytokine production profile. $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25(\text{OH})_2\text{D}_3$) has been reported to inhibit Th1/Tc1-related, but increase Th2/Tc2-associated cytokines in T cells from adults. In industrialized countries, vitamin D supplementation for prevention of rickets is initiated within the first days of life and continued throughout the entire first year. Epidemiologic studies suggest an association of vitamin D exposure in newborns with the incidence of allergic diseases in later life. This study addresses the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on Th1/Tc1 versus Th2/Tc2 differentiation in long term cell cultures of (naive) cord blood T lymphocytes. Our results show that in CD4^+ as well as CD8^+ cord blood cells, $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits not only IL-12-generated IFN- γ production, but also suppresses IL-4 and IL-13 expression induced by

IL-4. Thus, in cord blood $1\alpha,25(\text{OH})_2\text{D}_3$ induces a T cell population without predominance of Th2 related cytokines. (*Pediatr Res* 52: 12–18, 2002)

Abbreviations

CBMC, Cord blood mononuclear cells
PBMC, Peripheral blood mononuclear cells
IDC, Intracellular detection of cytokines
PHA, Phytohemagglutinin
PMA, Phorbol 12-myristate 13-acetate
PE, Phycoerythrin
APC, Allophycocyanin
PerCP, Peridinin chlorophyll
FSC, Forward Scatter
SSC, Side Scatter

Human naive T cells, which produce only IL-2, can differentiate into at least two functionally distinct subsets of memory or effector T cells, which can be distinguished by their cytokine profile (1, 2): CD4^+ Th1 cells on one hand producing predominantly IFN- γ , IL-2, and lymphotoxin, and CD4^+ Th2 cells on the other hand, which are the main source of IL-4 and IL-5, represent two extremely polarized phenotypes of a continuous spectrum of cytokine-producing T lymphocytes. Similar subpopulations (*i.e.* Tc1 and Tc2) exist in the CD8^+ T cell population (3, 4). Th1- and Th2-associated immune responses have been implicated in the pathogenesis of infectious, allergic, and autoimmune diseases (3).

The local cytokine environment plays an important role in the differentiation of naive T cells along the Th1/Tc1 or Th2/Tc2 pathway (5, 6). For example, *in vitro* priming of naive T cells in the presence of IL-4 generates IL-4-producing Th2 effector cells (7–10). Similarly, IL-12, secreted by monocytes

and dendritic cells, induces the capacity to produce IFN- γ in neonatal T cells (11).

Furthermore, the arachidonic acid metabolite prostaglandin E_2 (12, 13), and several members of the steroid hormone family, such as progesterone (14, 15), glucocorticoids (16), and $1\alpha,25$ -dihydroxyvitamin D_3 ($1\alpha,25(\text{OH})_2\text{D}_3$) (17) have been reported to influence Th and Tc subset generation. $1\alpha,25(\text{OH})_2\text{D}_3$ is not only required for normal mineral homeostasis but also regulates differentiation, growth, and function of different cell types such as hematopoietic and immune cells as well as a variety of normal and malignant epithelial cells (18–20). The discovery of the high affinity receptor for $1\alpha,25(\text{OH})_2\text{D}_3$ in monocytes and activated lymphocytes (21) suggested that this steroid acts as an immunoregulatory hormone (22, 23). Interference with cytokine production of monocytes and lymphocytes seems to be a key mechanism by which $1\alpha,25(\text{OH})_2\text{D}_3$ interacts with the immune system. Suppression of mitogen-induced proliferation (24) and inhibition of secretion of IL-1, IL-2, IL-6, IL-12, TNF, and IFN- γ have been observed in human blood mononuclear cells (PBMC) (25–30). In adult human CD4^+ and CD8^+ T cells, however, we have recently demonstrated the induction of IL-6-producing sub-

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populations by $1\alpha,25(\text{OH})_2\text{D}_3$ (31). Possible therapeutic implications have been deduced from the preferential inhibition of Th1-related cytokines by $1\alpha,25(\text{OH})_2\text{D}_3$ (17), which might prove useful in prevention of Th1-mediated clinical situations such as autoimmune reactions or of transplant rejection (32–34). In this respect, Wjst and Dold (35) have put forward the hypothesis that the association of vitamin D exposure in newborns with the incidence of allergic diseases in later life – suggested by a number of epidemiologic studies (36–38) – may be due to inhibition of Th1 differentiation and function by $1\alpha,25(\text{OH})_2\text{D}_3$. As vitamin D supplementation for prevention of rickets is initiated within the first days of life and continued throughout the entire first year, the influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on T cell cytokine production during differentiation of (naïve) cord blood T cells is of special interest. Although effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on cytokine production of T cells derived from peripheral blood of adults have been analyzed in several studies (31, 39–42), nothing is known about its action on neonatal cytokine production, which could be different from the adult situation, mainly because cord blood contains primarily naïve T cells expressing the CD45RA^+ phenotype (43).

In the present study the effects of $1\alpha,25(\text{OH})_2\text{D}_3$, alone or in concert with IL-4 and IL-12, on cytokine production and differentiation of cord blood CD4^+ and CD8^+ T cells were investigated using a flow cytometry-based intracellular cytokine detection method. We herewith report that in cord blood T cells, $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits not only Th1/Tc1 differentiation as in T cells from adults, but also suppresses Th2/Tc2 differentiation. $1\alpha,25(\text{OH})_2\text{D}_3$ specifically induces cord blood T cells to differentiate into a functionally distinct IL-6-producing subpopulation, which can be expanded through a permissive effect of IL-4. In addition we report a significant direct inhibitory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on $\text{IFN-}\gamma$ production of T cells, which is not mediated by suppression of IL-12 production by accessory cells.

MATERIALS AND METHODS

Cord blood mononuclear cells (CBMC). Cord blood was collected with a heparinized syringe by aspiration from the umbilical vein of four normal term deliveries. The mononuclear cells (CBMC) were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Culture conditions. CBMC ($10^6/\text{mL}$) were cultured in Ultra Culture (UC) Medium (Bio Whittaker, Walkersville, MD, U.S.A.) supplemented with 2 mM L-glutamine, and 170 mg/L gentamycin sulphate (both from Sigma Chemical Co. Bio Sciences, St. Louis, MO, U.S.A.). A schematic representation of the cell culture protocol is shown in Fig. 1: Initially PHA (Life Technologies, Grand Island, NY, U.S.A.) was supplemented at a concentration of 1% vol/vol. This was replaced by IL-2 (20 U/mL, Roche Diagnostics GmbH, Basel, Switzerland) after 3 d to maintain cell proliferation and viability. Cultures were exposed to $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M, kind gift from Hoffmann La-Roche, Basel, Switzerland), IL-4 (450 U/mL, Strathmann Biotech, Germany), or IL-12 (200 U/mL, gift from M. Gately, Hoffmann La-Roche, Nutley, NJ, U.S.A.), respectively, or to a combination of $1\alpha,25(\text{OH})_2\text{D}_3$ with either one of the two

Figure 1. Experimental protocol for CBMC culture. Duration of treatments is indicated by closed horizontal bars. Time points of medium change and addition of fresh reagents are indicated by vertical ticks. Vertical arrows indicate the time points when cultures were restimulated with PMA and ionomycin in the presence of monensin and subsequently processed for intracellular detection of cytokines (IDC).

cytokines. On day 7 and 14, cells were washed once and IL-2, $1\alpha,25(\text{OH})_2\text{D}_3$, IL-4 and IL-12 were re-added as described above. Fresh medium was added to the cultures on day 10 and 17. Cultures with IL-2 alone served as control.

Intracellular cytokine detection. On day 0, 7, 14, and 21, the percentage of cytokine-producing CD4^+ T helper cells (Th) and CD8^+ cytotoxic T cells (Tc) was analyzed by intracellular cytokine detection. Expression of $\text{IFN-}\gamma$, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 was assessed with MAb and four-color flow cytometry. The technique has been described previously (44, 45). At the respective time points, cells were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA), and 1.25 μM ionomycin, in the presence of 2 μM monensin (all from Sigma Chemical Co. Bio Sciences, St. Louis, MO, U.S.A.). 4 h later, cells were harvested, washed and fixed with 2% formaldehyde.

Four-color fluorescence staining was performed using rat or mouse anti-human MAb and the respective isotype controls labeled with FITC, phycoerythrin (PE), peridinin chlorophyll (PerCP), or allophycocyanin (APC): anti- CD4 (APC), anti- CD8 (PerCP), anti- $\text{IFN-}\gamma$ (FITC), anti-IL-2 (PE), anti-IL-4 (PE) (all from Becton Dickinson, San Jose, CA, U.S.A.), anti-IL-6-PE and anti-IL-13-PE (from Pharmingen, San Diego, CA, U.S.A.).

Statistics. Data were analyzed with the two-tailed paired Student's *t* test. *p*-Values <0.05 were considered significant.

RESULTS

Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on the frequency of cytokine- (IL-2, $\text{IFN-}\gamma$, IL-4, and IL-13) producing cord blood T cells. In the first series of experiments, cytokine production at the single cell level was assessed in the CD4^+ and CD8^+ fraction of cord blood mononuclear cells (CBMC), which had been cultured for up to 21 d in the absence or presence of 1×10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$. On day 0, both CD4^+ and CD8^+ cells, as expected, exclusively expressed IL-2, but were negative for all other cytokines under investigation ($\text{IFN-}\gamma$ – Fig. 2; IL-4, IL-6, IL-13 – data not shown).

The relative number of IL-2-producing cells within the CD4^+ population was similar in controls ($18.5 \pm 10.2\%$) and

Figure 2. Intracellular detection of cytokines in CD4⁺ and CD8⁺ human cord blood lymphocytes. Percentages of cytokine-producing cells were determined by four-color flow cytometry. Dot plots from one representative experiment (day 0) are shown. Left panel shows the forward/side scatter (FSC/SSC) plot of CBMC. Cells gated as lymphocytes are encircled. The middle panel shows gating on CD4⁺ and CD8⁺ lymphocytes. Dot plots of the right panel show specific cytokine-producing subpopulations of CD4⁺ and CD8⁺ lymphocytes, respectively. IL-2-positive/IFN- γ -negative cells are represented in the upper left, IL-2-negative/IFN- γ -positive cells are represented in the lower right quadrants. IL-2 and IFN- γ -co-expressing cells are shown in the upper right quadrants. The values within the quadrants represent means of 4 cord blood samples.

1 α ,25(OH) $_2$ D $_3$ -treated cultures ($17.6 \pm 6.9\%$) on day 7. However, on day 14, the percentage of IL-2 positive CD4⁺ cells was markedly lower in the latter ($53.5 \pm 9.4\%$ versus $28.8 \pm 18.5\%$; $p < 0.05$). The difference was no longer significant on day 21 ($43.8 \pm 8.5\%$ in controls versus $36.9 \pm 5.8\%$ in 1 α ,25(OH) $_2$ D $_3$ cultures) (data not shown).

The percentage of IFN- γ -producing cells was low in both the CD4⁺ and CD8⁺ subpopulation of untreated CBMC cultures. A significant ($p < 0.05$) inhibitory effect of 1 α ,25(OH) $_2$ D $_3$ was observed only in CD8⁺ cells on day 14 and day 21 (Fig. 3).

1 α ,25(OH) $_2$ D $_3$ had no effect on IL-4 production in CD4⁺ and CD8⁺ cells during a 21 day culture period, but transiently suppressed IL-13 production on day 14 ($p < 0.01$) (Fig. 4).

Effect of 1 α ,25(OH) $_2$ D $_3$ on induction of cytokine-(IFN- γ , IL-4, and IL-13) producing T cells by IL-4 or IL-12. When CBMC were cultured in the presence of IL-4, the frequency of IL-4-producing T cells had the tendency to rise, though only resulting in a significance for CD4⁺ cells on day 21 ($p < 0.05$). The fraction of CD4⁺ IL-13-positive cells was markedly, that of CD8⁺ IL-13-positive cells moderately, expanded by IL-4 (Fig. 4). This induction of IL-4 and IL-13-producing cells was almost completely inhibited by simultaneous addition of 1 α ,25(OH) $_2$ D $_3$ (Fig. 4).

As shown in Fig. 3, treatment of CBMC cultures with IL-12 increased the frequency of IFN- γ -expressing T cells in the CD4⁺ and in the CD8⁺ population. 1 α ,25(OH) $_2$ D $_3$ considerably reduced this effect. IL-12 also stimulated IL-4 production, which again was inhibited by 1 α ,25(OH) $_2$ D $_3$ (Fig. 3).

Modulation of 1 α ,25(OH) $_2$ D $_3$ -induced IL-6 production by IL-4 and IL-12. While control cultures remained negative for

Figure 3. 1 α ,25(OH) $_2$ D $_3$ and IL-12: time course of IL-4 and IFN- γ production by human CD4⁺ and CD8⁺ lymphocytes. PHA-stimulated CBMC were cultured with or without 1 α ,25(OH) $_2$ D $_3$, with IL-12, and with 1 α ,25(OH) $_2$ D $_3$ + IL-12. The percentage of cytokine producing cells was determined by four-color flow cytometry. Each column represents the mean $\pm$ SD from four experiments. Significance is expressed by p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

IL-6 throughout the observation period, addition of 1 α ,25(OH) $_2$ D $_3$ led to significant proportions of IL-6-expressing CD4⁺ ($6.7 \pm 4.1\%$, $p < 0.05$) and CD8⁺ ($6.1 \pm 3.7\%$, $p < 0.05$) lymphocytes on day 21. Neither IL-4 nor IL-12 had such stimu-

Figure 4. $1\alpha,25(\text{OH})_2\text{D}_3$ and IL-4: time course of IL-4 and IL-13 production by human CD4^+ and CD8^+ lymphocytes. PHA-stimulated CBMC were cultured with or without $1\alpha,25(\text{OH})_2\text{D}_3$, with IL-4, and with $1\alpha,25(\text{OH})_2\text{D}_3$ + IL-4. The percentage of cytokine producing cells was determined by four-color flow cytometry. Each column represents the mean + SD from four experiments. Significance is expressed by p values (* p < 0.05, ** p < 0.01, *** p < 0.001).

latory properties. IL-12 even tended to suppress this $1\alpha,25(\text{OH})_2\text{D}_3$ effect when supplemented simultaneously, though without reaching statistical significance (Fig. 5).

In contrast, the combination of IL-4 and $1\alpha,25(\text{OH})_2\text{D}_3$ accelerated and amplified the surge of IL-6-positive T cells so that already on day 14 a significant proportion of both CD4^+ and CD8^+ cells expressed IL-6. On day 21, the percentage of IL-6-positive CD4^+ cells was three times higher than in the cultures treated with $1\alpha,25(\text{OH})_2\text{D}_3$ alone (Fig. 5). A significant IL-4-related increment in the IL-6 levels was also noted in CD8^+ cells (Fig. 5).

$1\alpha,25(\text{OH})_2\text{D}_3$ specifically modulates cytokine (co-) expression patterns triggered by IL-4 or IL-12. Co-expression of IFN- γ with IL-6, IL-4 or IL-13 after 21 d of culture with IL-4 or IL-12 +/- $1\alpha,25(\text{OH})_2\text{D}_3$ is presented in Fig. 6 for CD4^+ and in Fig. 7 for CD8^+ lymphocytes. In CD4^+ cells, IL-12 generates a Th1 pattern, defined by predominance of a subpopulation of cells only expressing IFN- γ (Fig. 6). Also cells positive for IL-4 or IL-13 frequently co-express IFN- γ (upper right quadrants). Simultaneous incubation with IL-12 and $1\alpha,25(\text{OH})_2\text{D}_3$ reduces the frequency of IFN- γ -producing lymphocytes. Under this condition, IL-6-positive cells appear to partially co-express IFN- γ .

IL-4 induces a typical Th2 phenotype, characterized by a significant proportion of cells producing only IL-4 and IL-13, but scanty IFN- γ and no IL-6 (Fig. 6). The most remarkable phenotype evolves in cultures treated with IL-4 and $1\alpha,25(\text{OH})_2\text{D}_3$ which is characterized by a significant subpopulation of T cells only positive for IL-6, and a decrease of the percentages of IL-4, IL-13 and IFN- γ -expressing cells (Fig. 6). Although total rates of IL-4 and IL-13-positive cells (Fig. 6, upper left plus upper right quadrants) are comparable to those from cultures with IL-12,

Figure 5. $1\alpha,25(\text{OH})_2\text{D}_3$ and IL-4 or IL-12: time course of IL-6 production by human CD4^+ and CD8^+ lymphocytes. PHA-stimulated CBMC were cultured with or without $1\alpha,25(\text{OH})_2\text{D}_3$, with IL-4 or IL-12, and with $1\alpha,25(\text{OH})_2\text{D}_3$ + IL-4 or IL-12. Percentage of IL-6-producing cells was determined by four-color flow cytometry. Each column represents the mean + SD from four experiments. Significance is expressed by p values (* p < 0.05, ** p < 0.01, *** p < 0.001).

co-expression of IFN- γ and IL-4 or IL-13 (upper right quadrants) is more pronounced (p < 0.05) in IL-12 cultures.

In CD8^+ cord blood cells (Fig. 7) regulatory effects on cytokine (co-)expression patterns are similar to CD4^+ cells.

Figure 6. $1\alpha,25(\text{OH})_2\text{D}_3$ modulates *in vitro* triggered Th1/Th2 cytokine production patterns. PHA-stimulated CBMC were cultured for 21 d with the appropriate cytokine and/or $1\alpha,25(\text{OH})_2\text{D}_3$ (D3). CD4^+ lymphocytes were gated as shown in Fig. 2. In all dot plots, x axis reflects IFN- γ expression, whereas y axis shows IL-6 (first panel), IL-4 (second panel) or IL-13 (third panel) production. Under culture conditions with IL-12, naive CD4^+ T cells differentiated into a typical Th1 phenotype (left column), whereas with IL-4, differentiation into a typical Th2 phenotype was observed (right column). These polarized phenotypes were modified by simultaneous addition of $1\alpha,25(\text{OH})_2\text{D}_3$, as shown in the two central columns (D3+IL-12 and D3+IL-4). Dot plots from one representative experiment are shown. Values within quadrants represent means of four experiments.

However, the CD8⁺ population generally comprises higher proportions of IFN- γ -positive cells, and lower percentages of cells producing IL-4 and IL-13. Also, IL-6-producing cells are less frequent in the CD8⁺ than in the CD4⁺ population.

DISCUSSION

1 α ,25(OH)₂D₃ represents an immunoregulatory hormone with distinct immunosuppressive activities, inhibiting T cell proliferation and Th1-associated cytokine production (25, 33, 39, 46, 47). Th1 inhibition has been suggested to trigger a Th2-associated immune response (17). Furthermore, we have recently shown that 1 α ,25(OH)₂D₃ has the capacity to enhance the development of cells producing Th2/Tc2 associated cytokines without impairing IFN- γ production (31).

All studies in the human system published so far have investigated cells derived from adult peripheral blood, which contains predominantly mature T cells. In our present experiments, umbilical cord blood T cells were used as a source of immunologically naive cells (43). Our data show that 1 α ,25(OH)₂D₃ suppresses Th1/Tc1 as well as Th2/Tc2-associated cytokine production driven by IL-12 or IL-4, respectively. This suggests that 1 α ,25(OH)₂D₃ modulates Th/Tc differentiation in memory and naive T cells in different ways.

Several investigators assume an indirect mechanism for the suppression of IFN- γ production by 1 α ,25(OH)₂D₃, *i.e.* mediated by inhibition of IL-12 secretion from costimulatory cells, such as monocytes, dendritic cells, or B cells (30, 48). In our experiments, the addition of 1 α ,25(OH)₂D₃ to IL-12-treated

cord blood T cells considerably reduced the proportion of IFN- γ -producing CD4⁺ and CD8⁺ T cells, suggesting a direct inhibitory effect as well.

IL-6 is a multifunctional cytokine involved in the regulation of acute phase reaction and hematopoiesis (49), which induces B cell differentiation to antibody producing plasma cells (50, 51) and regulates T cell growth and differentiation (52–54). Although initially thought to be a pro-inflammatory cytokine, there is increasing evidence supporting a predominantly anti-inflammatory and immunosuppressive role of IL-6 and of some of the IL-6-regulated acute phase proteins (55).

In human peripheral blood, monocytes represent the primary and main source of IL-6 (56). In addition, mitogen-stimulated T cells have been demonstrated to produce IL-6 (57, 58). 1 α ,25(OH)₂D₃ can have variable effects on IL-6 production by human PBMC. Depending on the cell stimulus, inhibition as well as induction or increase of IL-6 has been measured in cell culture supernatants. 1 α ,25(OH)₂D₃ seems to suppress IL-6 release by monocytes (27, 47) while stimulating IL-6 producing T cells (31, 42). Our results from the experiments with naive cord blood T cells are in accordance with those obtained with adult T cells (31). PBMC, activated in the presence of 1 α ,25(OH)₂D₃ develop a significant proportion of IL-6-positive CD4⁺ and CD8⁺ T cells. While, naive T cells require a culture period of more than 14 d till measurable IL-6 expression takes place, a significant proportion of IL-6-positive T cells could be detected already on day 7 in samples from adults (31). Naive (CD45RA⁺) T cells from peripheral blood of adults show delayed responses to 1 α ,25(OH)₂D₃, compared with the mature (CD45RO⁺) T cell fraction (59), and cord blood CD45RA⁺ T cells fail to acquire similar activation status to their adult counterparts (60).

Two studies indicate that IL-4 synergizes with 1 α ,25(OH)₂D₃ on leukemia cells (61, 62). IL-4 has been described to potentiate the pro-differentiating and anti-proliferative effect of 1 α ,25(OH)₂D₃ in myeloid and monocytic leukemia cells of mice (16, 63). Our data indicate that this synergistic effect also operates in T cells. For the latter, we demonstrated a significant concurrent antagonism of 1 α ,25(OH)₂D₃ and IL-4 in respect to IL-4 and IL-13 production. This indicates that the IL-6-positive T cell subpopulation represents a specific phenotype selectively induced and enhanced by particular stimuli.

Beside a Th1/Tc1 or Th2/Tc2 phenotype a Th3 subset has been described, mainly characterized by its TGF- β production, with varying amounts of IL-4 and IL-10 (64). The discovery of these cells once more suggests the existence of polarized T cell subsets beyond the Th1/Th2 dichotomy. Until now intracellular detection of TGF- β by flow cytometry is technically not possible. However, we have analyzed production of IL-10, which we could not detect in any cord blood cultures treated with IL-4 plus 1 α ,25(OH)₂D₃ (data not shown). Moreover, there are no reports about pronounced IL-6 production by Th3 cells so far. Therefore, we suggest that the IL-6-producing T cell subpopulation is distinct from the Th3 subset as well.

It has been shown that only IL-4 but not IL-6 generates IgE antibodies (65). Furthermore, IL-6 induces B cells in PBMC to become IgG- and IgA-producing cells (66, 67). IgG1 response

Figure 7. 1 α ,25(OH)₂D₃ modulates *in vitro* triggered Tc1/Tc2 cytokine production patterns. PHA-stimulated CBMC were cultured for 21 d with the appropriate cytokine and/or 1 α ,25(OH)₂D₃ (D3). CD8⁺ lymphocytes were gated as shown in Fig. 2. In all dot plots, x axis reflects IFN- γ expression, whereas y axis shows IL-6 (first panel), IL-4 (second panel) or IL-13 (third panel) production. Under culture conditions with IL-12 naive CD8⁺ T cells differentiated into a typical Tc1 phenotype (left column), whereas with IL-4, differentiation into a typical Tc2 phenotype was observed (right column). These polarized phenotypes were modified by simultaneous addition of 1 α ,25(OH)₂D₃, as shown in the two central columns (D3+IL-12 and D3+IL-4). Dot plots from one representative experiment are shown. Values within quadrants represent means of four experiments.

was stronger than IgG2 and IgG4 antibody production (67). The synergistic induction of an IL-6-producing subpopulation with partial inhibition of IL-4-positive T cells by simultaneous exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ and IL-4 may thus modify immunoglobulin isotypes relations.

The reported preferential inhibition of Th1/Tc1-like cytokines by $1\alpha,25(\text{OH})_2\text{D}_3$ raised fear of boosting toward Th2/Tc2-related immune responses, favoring development of allergy (35). However, our data demonstrate a balanced effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on Th1/Tc1 and Th2/Tc2 cytokines during differentiation of cord blood T cells, suggesting that in the perinatal period application of vitamin D should not promote the development of allergies.

It is remarkable that $1\alpha,25(\text{OH})_2\text{D}_3$ regulates cytokine production in cord blood CD4^+ as well as CD8^+ T cells in parallel. In this respect our data are in line with results from adult blood T cells (31) and with reports that CD8^+ T cells are able to produce Th1- and Th2-type cytokines. The differentiation of CD8^+ cells into Tc1 and Tc2 is triggered by the same agents necessary for Th1 and Th2 development (45, 68–70).

We have previously shown that IFN- γ , IL-4, or IL-13 co-expression of IL-6-positive CD4^+ and CD8^+ T cells is negligible (31). In the present study a divergent regulation of Th2/Tc2-like cells compared with IL-6-producing T cells could be demonstrated, strengthening the hypothesis that these cells represent a specific subset outside the continuum of Th1/Tc1 and Th2/Tc2 phenotypes.

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