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The Effects of 1,25-Dihydroxyvitamin D₃ on In Vitro Human NK Cell Development from Hematopoietic Stem Cells

Matthew A. Weeres,* Kim Robien,† Yong-Oon Ahn,* Marie-Luise Neulen,* Rachel Bergerson,* Jeffery S. Miller,‡ and Michael R. Verneris*

1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the biologically active form of vitamin D and is immunoregulatory. 1,25(OH)₂D₃ binds the vitamin D receptor complex present in many immune populations and can illicit transcriptional responses that vary among different immune subsets. The effects of 1,25(OH)₂D₃ on mature and developing human NK cells are not well characterized. In the present study, we examined the influence of 1,25(OH)₂D₃ using an established NK cell differentiation system. Briefly, umbilical cord blood CD34⁺ cells were isolated and cultured in conditions optimal for NK cell differentiation, and varying concentrations of 1,25(OH)₂D₃ were administered. At physiological concentrations (10 nM), 1,25(OH)₂D₃ impaired NK cell development. Moreover, the NK cells that did develop under the influence of 1,25(OH)₂D₃ showed a significant reduction in function (cytotoxicity and cytokine production). Conversely, 1,25(OH)₂D₃ strongly induced hematopoietic stem cells to differentiate along a myeloid pathway, giving rise to CD14⁺ cells. Mechanistically, 1,25(OH)₂D₃ drives hematopoietic progenitor cells to rapidly upregulate monocyte genes (i.e., C/EBP-α and CD14). There were no effects of 1,25(OH)₂D₃ on mature NK cytotoxicity or cytokine production. Collectively, these studies provide novel data showing the negative regulatory effect of 1,25(OH)₂D₃ on NK cell development. The Journal of Immunology, 2014, 193: 3456–3462.

The fat-soluble prehormone 25(OH)D is produced in the liver and can be converted to its active form [1,25(OH)₂D], where it regulates calcium metabolism and skeletal health by stimulating gastrointestinal calcium absorption, thereby promoting bone mineralization. Evidence for the role of vitamin D intake on health first came from studies on rickets (1). Vitamin D deficiency is also associated with the development of cardiovascular diseases, cancer, and autoimmune disorders (2). Extensive media coverage of the potential health benefits of vitamin D supplementation has translated into steady increases in vitamin D intake by the public. Accordingly, sales of vitamin D supplements in the United States have increased from $75 million in 2006 to $550 million in 2010, suggesting that large numbers of individuals are using these supplements. Given the increased usage, research is needed to better understand the benefits, as well as the risks, of vitamin D supplementation. These issues could be considered a matter of both consumer protection and public health.

There has been increasing recognition that the active form of vitamin D [1,25-dihydroxyvitamin D₃, or 1,25(OH)₂D₃] impacts the immune system. For instance, 1,25(OH)₂D₃ has potent anti-proliferative activity on T cells after mitogen activation through the upregulation of inhibitory ligand receptors such as CTLA-4 (1, 3). Inhibition of proliferation in lymphoid and myeloid leukemia cell lines is also seen at the level of cell cycle regulation, as 1,25(OH)₂D₃ upregulates p21 and p27 proteins and downregulates CDK2/4, cyclin D1, and cyclin A (3–5). In addition to inhibiting proliferation, 1,25(OH)₂D₃ also activates proapoptotic pathways by downregulating BCL2, thereby sensitizing lymphocytes to apoptosis (6, 7). Vitamin D has also been shown to skew T cells to a less inflammatory state. For instance, 1,25(OH)₂D₃ decreases T cell IFN-γ production and increases IL-4 production (8). Both the generation and immune suppressive capacity of Foxp3⁺ CD4 regulatory T cells are increased by 1,25(OH)₂D₃ (5, 9). More recent studies also show that 1,25(OH)₂D₃ prevents T cells from producing the inflammatory cytokine IL-17 (6, 10). In line these results, other groups have documented that 1,25(OH)₂D₃ negatively modulates development of Th17 cells (6). Physiologically relevant doses of 1,25(OH)₂D₃ also inhibit the production of IL-17, IL-21, and IL-22 in Th17-skewed T cells, suggesting that major transcription changes are driven by the vitamin D receptor transcription factor complex.

NK cells are innate immune effector cells that play a crucial role in both tumor and viral surveillance (11). Unlike T or B cells, which express a single germline rearranged Ag receptor, NK cells clonally display a diverse repertoire of both activating and inhibitory receptors that recognize aberrant cells that have lost MHC class I expression or acquired stress receptors that trigger NK cell activation (12). NK cells are the first lymphocyte population to recover after allogenic transplantation, potentially linking these cells to the early graft-versus-leukemia reactions that occur after allogeneic transplantation. Using heavy water labeling, prior studies show that human NK cells disappear from the peripheral circulation relatively rapidly (6.9%/d; 1/2 of <10 d). Thus, unlike T or B cells, which are thought to be long-lived, NK cells need to be replenished constantly by hematopoietic stem cells (HSCs) (13). The effects of 1,25(OH)₂D₃ on mature NK cell proliferation...
and modulation of functional activity have been previously explored. Contrary to the above studies on T cells, the literature regarding the impact of 1,25(OH)2D3 on NK cells has been varied and ranges from augmentation of NK cell proliferation and cytotoxicity to an inhibition in these activities (14–16). The extent to which 1,25(OH)2D3 affects NK cell development from CD34+ umbilical cord blood (UCB) stem cells remains understudied.

In both mice and humans, NK cells have been shown to undergo a series of developmental intermediates that can be identified on the basis of cell surface receptors (17, 18). These intermediates vary anatomically and functionally. For instance, in humans, the earliestNK progenitors (NKp) are stage II NK cells and are identified by the surface phenotype CD34+CD117highCD45RA+. These cells likely emerge from the bone marrow and traffic to secondary lymphoid tissues, such as the lymph nodes, where they subsequently differentiate to stage III NKp (CD56+CD117highCD94−) under the influence of instructive cytokines, including IL-15. Stage III progenitors lack NK functionality (i.e., the ability to produce INF-γ or kill tumor targets). Through an undefined process stage III NK cells develop into stage IV NK cells (CD56brightCD117highCD94−) where they acquire the ability to produce IFN-γ in response to cytokine stimulation (IL-12 and IL-18) and have attenuated cytotoxicity. Following this, stage IV NK cells are released from the secondary lymphoid tissues to further differentiate into mature stage V NK cells (CD56dimCD16+CD94+/−KIR−), which have potent cytotoxicity and cytokine production. We have previously reported that UCB CD34+ progenitors cultured with cytokines (IL-15, IL-3, IL-7, FLT3 ligand, and stem cell factor [SCF]) and a fetal liver stromal cell line can differentiate into functional stage IV and V human NK cells during the course of 28 d. This model closely mirrors the developmental stages described in primary human secondary lymphoid tissues (18, 19).

To study the role of vitamin D on NK cell development, physiologically relevant concentrations of 1,25(OH)2D3 (10 nm) were added to the NK differentiation cultures. In conditions that contained 1,25(OH)2D3 there was a significant reduction in overall cell expansion and a marked reduction of NK differentiation. Of the cells that did differentiate into NK cells, they were immature and had reduced function. The reduction in NK cell differentiation was further explained by an increase in the number of CD14+ monocytes differentiating from CD34+ stem cells, despite conditions that are optimal for NK cell differentiation. Stem cells cultured in 1,25(OH)2D3 rapidly upregulated monocyte-associated genes, including C/EBP-α and CD14 within 72 h. Interestingly, there was no impact of 1,25(OH)2D3 on short-term (7 d) cultures of mature, peripheral blood NK cells that were tested for cytotoxicity or IFN-γ production. Collectively, these results show that 1,25(OH)2D3 favors monocyte development at the cost of NK cells.

Materials and Methods

Isolation of CD34+ cells from UCB and CD56+ cells from peripheral blood

After Ficoll separation, CD34+ progenitor cells were isolated from UCB using magnetic bead selection (Miltenyi Biotec, Auburn, CA). Selected cells were routinely >90% pure. CD3+ CD56+ cells were isolated from human peripheral blood using the Rosette separation method according to the manufacturer’s specifications (StemCell Technologies, Vancouver, BC, Canada). Cells were tested for purity and were 85–90% pure.

Culture of the stromal cell line EL08.1D2

The embryonic liver cell line EL08.1D2 was cultured on gelatinized plates at 32°C in 40.5% α-MEM (Invitrogen, Carlsbad, CA), 50% Myelocult (M5300; StemCell Technologies), 7.5% FBS with 2-ME (50 μM/L), GlutaMAX (2 mM), penicillin (100 U/ml)/streptomycin (100 U/ml), and hydrocortisone (10−6 M). Prior to coculture with progenitor cells, EL08.1D2 cells were irradiated (3000 rad).

NK cell differentiation cultures

CD34+ selected cells (500/well) were plated in a 24-well plate on an irradiated confluent monolayer of EL08.1D2 cells in Ham F12 plus DMEM (1:2 ratio) with 20% male, human AB− sera (SeraCare Life Sciences, Oceanside, CA), ethanolamine (50 μM), ascorbic acid (20 μg/ml), 5 μg/ml sodium selenite, 2-ME (24 μM), and penicillin (100 U/ml)/streptomycin (100 U/ml). At the start of cultures, IL-3 (5 ng/ml), IL-7 (20 ng/ml), IL-15 (10 ng/ml), SCF (20 ng/ml), and FLT3 ligand (10 ng/ml) were added along with equal volumes of either a vehicle control (100% ethanol) or 1,25(OH)2D3 (Sigma-Aldrich). Cultures were refreshed by semi-depletion (50% volume change) supplemented with the previously mentioned cytokines except IL-3. The number of cells was determined in each condition on days 14, 21, and 28.

FACS staining and mAbs

The following Abs were used: CD34 (PerCP-Cy5.5 or PE, clone 581), CD56 (PerCP-Cy5.5 or PE-Cy7, clone B159), CD94 (FITC, clone HP3E4), CD117 (PE-Cy7, clone 104D2 or PerCP-Cy5.5, clone YB5.B8), CD161 (FITC, clone DX12), CD14 (allophycocyanin-Cy7, clone M4p9), CD158a (FITC clone, HPE34), CD158b (FITC, clone CH-2), and NKp44 (FITC, clone DX9), all from BD Biosciences (San Jose, CA). Additional Abs included NK2D2 (PE, clone 149810), NKp30 (PE, clone 210845), CD158a (FITC clone, HPE34), CD158b (FITC, clone CH-2), and NKp44 (PE, clone 253415), and NKp46 (PE, clone 195314), all obtained from R&D Systems (Minneapolis, MN). Intracellular staining for IFN-γ (PE clone 45.B3) was performed using CytOx/Cytoperm (BD Biosciences). IFN-γ staining was performed after 16 h of stimulation with IL-12 (10 ng/ml) and IL-18 (100 ng/ml). Brefeldin A was added for the last 4 h. Data were analyzed using FlowJo version 7.6. FACS sorting was performed on either a FACS Vantage or FACS Aria (BD Biosciences).

35S-Cr-release assay

The immortalized erythroleukemia cell line K562 was used as an NK target for cytotoxicity and was labeled with 35S (DuPont-NEN Research Products, Boston, MA) by incubating 1 × 107 cells in 11.1 MBq (300 μCi) 35S for 1 h at 37°C, 5% CO2. The cells were washed with PBS, resuspended in RPMI 1640 with 10% FBS, and plated in 96-well plates at 1 × 104 cells/well in triplicate. Effector cells were added at specified ratios (5:1 to 1:2.5:1) and incubated for 4 h at 37°C, 5% CO2. Supernatants were collected and counted (Wizard 1470, PerkinElmer, Shelton, CT). Specific 35S lysis was calculated using the equation: % specific lysis = 100 × [(test release − spontaneous release)/(maximal release − spontaneous release)].

RNA sequencing analysis

UCB CD34+ HSCs were freshly isolated from three different donors and used for NK cell differentiation (as described above) with either 1,25(OH)2D3 (10 nM) or vehicle control (100% ethanol). After 72 h the cells were harvested and RNA was isolated using an RNeasy Mini kit (Qiagen). Samples were assessed by RNA sequencing analysis (Illumina HiSeqation 2000) at the Minnesota Biomedical Genomics Center (Minneapolis, MN). Data were analyzed for differences in fold change of gene expression using the Partex Genomics Suite version 6.6 software. Statistical analysis

Differences between the various conditions were determined using GraphPad Prism software version 5 with either a t test or ANOVA as appropriate.

Results

1,25(OH)2D3 suppresses overall cell expansion, percentage of CD56+ cells, and absolute number of NK cells

To investigate the influence of vitamin D on NK cell differentiation and maturation, physiological concentrations of 1,25(OH)2D3 (10 nM) were added to NK cell differentiation cultures. Briefly, CD34+ cells were cultured on a stromal line (EL08.1D2) and in the presence of IL-3, SCF, FLT3 ligand, IL-7, and IL-15. As we have previously demonstrated, these conditions are optimal for NK cell differentiation and give rise to ~2000- to 3000-fold NK cell expansion from a single stem cell during the course of 21–
28 d (19, 20–22). As shown in Fig. 1A, 1,25(OH)2D3 significantly decreased the number of mononuclear cells compared with the vehicle controls (p = 0.01). Examining the cultures on days 14, 21, and 28 showed that a significantly lower percentage of NK cells was present in 1,25(OH)2D3-containing cultures compared with the vehicle control (Fig. 1B, p < 0.01). Combining the above information showed that the total numbers of NK cells that developed in the presence of 1,25(OH)2D3 were significantly less than in vehicle controls (Fig. 1C, p < 0.01). Thus, under conditions that are optimal for NK cell differentiation (19, 20–22), 1,25(OH)2D3 markedly inhibits NK development.

Effect of supplemental 1,25(OH)2D3 on the function of stem cell–derived NK cells

Although significantly fewer NK cells develop in the presence of 1,25(OH)2D3, the functions of these cells are unknown. On days 21 and 28 of differentiation, 51Cr-release assays were performed to compare the cytotoxic capacity of NK cells that differentiated in the presence of 1,25(OH)2D3 to the vehicle controls. NK cells were purified from cultures and used to kill the prototypic NK target, K562 cells. As shown in Fig. 2A, purified HSC-derived NK cells that developed in the presence of 1,25(OH)2D3 showed a reduction in cytotoxicity compared with the vehicle control group (p < 0.05, average of n = 8 donors). We also investigated the potential changes in the ability of NK cells to produce INF-γ following stimulation with monokines (IL-12 and IL-18). As shown in Fig. 2B, the percentage of INF-γ–producing NK cells was decreased in NK cells that developed in the presence of 1,25(OH)2D3 (p = 0.02, average of n = 4 donors). These results could be explained by either slower NK differentiation (see below), impaired function, or both. To address the effect of 1,25(OH)2D3 on function, mature NK cells (stages IV and V) were purified from differentiation cultures on the basis of CD94 expression (which marks stage IV and V NK cells). Purified CD94+ NK cells that were differentiated in the presence of 1,25(OH)2D3 were less cytotoxic than controls (Fig. 2C, p = 0.02, n = 4). Collectively, the above studies show that HSC-derived NK cells differentiated in the presence of 1,25(OH)2D3 show a marked attenuation of both cytotoxicity and cytokine production.

FIGURE 1. Vitamin D reduces the number of CD34-derived NK cells. (A) Fold expansion of MNCs following culture of CD34+ HSCs in the presence of 1,25(OH)2D3 or vehicle control. On days 14, 21, and 28 cells were enumerated using trypan blue staining. (B) The percentage of CD56+ cells as determined by FACS on days 14, 21, and 28 of culture. (C) The absolute numbers of NK cells on days 14, 21, and 28 of culture with vehicle or 1,25(OH)2D3. Shown are the average results of 10 donors (p < 0.01).
Effect of 1,25(OH)2D3 on NK differentiation

The developmental stage of NK cells can be determined using CD117 and CD94 (8, 12). Specifically, stage III NK progenitors lack cytotoxicity and cytokine production and are marked by a CD56+CD117highCD94− phenotype. During differentiation, stage III NK progenitors downmodulate CD117 and acquire the CD94/NKG2A inhibitory receptor while progressing to the next developmental stage (stage IV, CD56+CD117int/lowCD94+) (12). At day 28, most cells in the control groups are stage IV, but when 1,25(OH)2D3 is present, more stage III NK progenitors are noted (Fig. 3). Thus, 1,25(OH)2D3 also delays NK maturation.

1,25(OH)2D3 drives monocyte differentiation at the expense of NK cells

In the presence of 1,25(OH)2D3 the percentage and absolute numbers of CD56+ cells were significantly less than in controls (Fig. 1). In the 1,25(OH)2D3-treated samples compared with controls (p < 0.01) (Fig. 4A), which was consistent across a series of donors (n = 4) (Fig. 4B). This information, coupled with a lower fraction of NK cells, suggests that 1,25(OH)2D3 induces monocyte differentiation at the cost of NK development.

1,25(OH)2D3 acts on early progenitors to inhibit NK development

The above studies show that 1,25(OH)2D3 inhibits NK cell development, but it was unclear where in development this was acting. To investigate this, 1,25(OH)2D3 was added at varying times of culture (days 0, 4, 7, 12, and 16). The total numbers of cells were dramatically reduced when 1,25(OH)2D3 was added at early time points (days 0, 4, and 7) (Fig. 5A); however, at later times (days 12 and 16), there was no impact on total cell number. Similarly, the percentage of NK cells was decreased when 1,25(OH)2D3 was added at the early time points (days 0, 4, and 7), but not when it was added at later times (days 12 and 16, Fig. 5B). Collectively, these results show that 1,25(OH)2D3 acts upon early progenitors, significantly impairing NK development.

Impact of 1,25(OH)2D3 on gene expression in early progenitors

Based on the above data showing that monocytes develop in the presence of 1,25(OH)2D3 (Fig. 4) and that this occurs early in development (Fig. 5), we hypothesized that 1,25(OH)2D3 initiates a genetic program in early progenitors that favors monocyte development. Given that we could detect CD14+ cells as early as day 7 after the start of cultures (not shown), we analyzed the changes in gene expression using an RNA sequencing array analysis at day 3 of culture in either vehicle or 1,25(OH)2D3 to identify genes that were differentially expressed in early progenitors. As shown in Table I, relatively few genes were significantly altered in the presence of 1,25(OH)2D3 (defined as >1.5-fold over control). Interestingly, CD14 was the only gene to change >2.5-fold, reinforcing the notion that 1,25(OH)2D3 drives developing progenitors to differentiate toward the monocyte lineage. Other monocyte-associated genes that were unregulated during the first 72 h of culture with 1,25(OH)2D3 include Siglec-12 (CD33 family member), C/EBP-α, and C/EBP-δ.

1,25(OH)2D3 has minimal impact on the expansion and cytotoxicity of human peripheral blood NK cells

We next set out to test the effects of 1,25(OH)2D3 on NK cells isolated from peripheral blood. NK cells were purified by negative selection and used for a 51Cr-release assay. Results are shown as the average ± SD of eight individual donors (p < 0.05). (B) Purified NK cells were stimulated for 16 h with IL-12 (10 ng/ml) and IL-18 (100 ng/ml) and cells were assessed for IFN-γ production using intracellular cytokine staining (n = 4, p = 0.03). (C) Function of purified stage IV and V NK cells differentiated with 1,25(OH)2D3 or vehicle control. Cells were cultured as described and then purified based on CD94 expression and used in a 51Cr-release assay. Shown are the individual results of four separate CD34+ donors used to generate NK cells (p = 0.02).
selection and cultured for 7 d with media supplemented with IL-15 (10 ng/ml) with either 1,25(OH)2D3 or vehicle control. Unlike the effects of 1,25(OH)2D3 on HSC-derived NK cells, there was no significant changes in either NK cell expansion (Fig. 6A, \(p = 0.06, n = 4\)), cytokine production (Fig. 6B, \(p = \text{ns}, n = 4\)), or cytotoxicity (Fig. 6C, \(p = \text{ns}, n = 8\)).

**Discussion**

In this study, we assessed the impact of 1,25(OH)2D3 on the development of NK cells using a validated NK cell differentiation model (20). This optimized system is highly robust, yielding \(\sim 2000\)–3000-fold expansion of functional NK cells from a single stem cell (19, 20). In the present study, we show that physiologic concentrations of 1,25(OH)2D3 inhibit the overall cell expansion and cause the developing progenitors to shift away from differentiating into the NK lineage. Although significantly reduced in number, the NK cells that do differentiate in the presence of 1,25(OH)2D3 are less mature and show a reduction in NK function, with less IFN-\(\gamma\) production and cytotoxicity. Interestingly, we found no differences in the expression of NK-activating receptors, including NKG2D, NKp30, and NKp46 (not shown), suggesting that the role of 1,25(OH)2D3 in attenuating function lies at a deeper level than recognition and activation of surface receptor/ligand interactions. Surprisingly, despite conditions that have been optimized for NK cell development, the addition of 1,25(OH)2D3 led to rapid commitment of hematopoietic progenitor cells to the myeloid lineage with significant monocyte development.

Lineage specification during hematopoiesis is a complex process where stem and progenitor cells integrate external signals from cytokine receptors and surface proteins. Triggering of these receptors feed into signaling cascades that activate transcription factors to drive lineage-restricted genetic programs, specifying developmental fates. A variety of transcription factors have been shown to be critical for NK cell differentiation, including E4BP4, ID family transcription factors, EOMES, and T-bet (reviewed in Ref. 23). By altering the time that 1,25(OH)2D3 was added to the...
administration of 1,25(OH)2D3 has previously been shown to increase proximal regions of C/EBP family genes, and exogenous admin-
ing, vitamin D–responsive elements have been identified in the heterodimers and drive myeloid differentiation (24, 25). Interest-

ation, including C/EBP-
some of these have clearly been implicated in myeloid specifi-
small number of genes were altered by 1,25(OH)2D3. However, 1,25(OH)2D3 was present, suggest that 1,25(OH)2D3 acts early in

In the presence of IL-3, IL-6, and SCF or GM-CSF, stem cells acquire their unique properties in the uterus, but it is interesting to note that vitamin D is produced by trophoblasts (37, 38) and that vitamin D deficiency is a risk factor for recurrent fetal loss after in vitro fertilization and embryo implantation (37, 39). Considering a dynamic cross-talk between NK cells and tropho-

Table I. Changes in gene expression progenitors cultured in NK differentiation cultures in 1,25(OH)2D3

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Fold Change</th>
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<td>+2.12</td>
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<td>+2.11</td>
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<td>SIGLEC-12</td>
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<tr>
<td>IL-8</td>
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<td>DOK1</td>
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<td>OSM</td>
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<tr>
<td>Galectin-1</td>
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</tr>
<tr>
<td>Vimentin</td>
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Purified CD34+ cells were cultured on EL08.1D2 cells as described in Materials and Methods in the presence of IL-3, IL-7, IL-15, SCF, and FLT3 ligand with 1,25(OH)2D3 or vehicle for 72 h and cells were collected for RNA isolation and RNA-sequencing analysis.

cultures, we observed dramatically different developmental fates of stem cells that were otherwise treated identically. Adding 1,25(OH)2D3 at the start of the culture strongly inhibited NK development, whereas delaying the introduction of 1,25(OH)2D3 by just 3 or 7 d diminished both the percentage and absolute numbers of NK cells that differentiated. In sharp contrast, adding 1,25(OH)2D3 at later times (≥12 d) had no major impact on NK cell development. These results, coupled with the rapid appearance of CD14+ cells (within 7 d of the start of cultures) when 1,25(OH)2D3 was present, suggest that 1,25(OH)2D3 acts early in differentiation to skew progenitors away from the NK lineage. RNA sequencing analysis on HSCs cultured in NK differentiation conditions with and without 1,25(OH)2D3 showed that a strikingly small number of genes were altered by 1,25(OH)2D3. However, some of these have clearly been implicated in myeloid specifi-
cation, including C/EBP-α and C/EBP-β, which form homo- or heterodimers and drive myeloid differentiation (24, 25). Interest-

ing, vitamin D–response elements have been identified in the proximal regions of C/EBP family genes, and exogenous admin-
istration of 1,25(OH)2D3 has previously been shown to increase the expression of one of the family members, C/EBP-β (26). The C/EBP family of transcription factors is especially important in monocyty development, supporting our findings (27). In our

studies there was no decrease in the expression of transcription factors involved in NK differentiation, perhaps suggesting that genes such as C/EBP-α and C/EBP-β dominate over NK-associated transcription factors to drive monocyte differentiation. In the presence of IL-3, IL-6, and SCF or GM-CSF, stem cells with ectopic expression of C/EBP-α show monocyte development (27), conditions similar to ours. The gene that was most strongly upregulated by 1,25(OH)2D3 in our studies was CD14, again consistent with existing literature showing that C/EBP-α and C/EBP-β bind to the CD14 promoter to transactivate gene expression (28). Interestingly, other investigators have examined the impact of 1,25(OH)2D3 on the ability of monocytes to acquire functions of dendritic cells (after addition of GM-CSF and IL-4 and inflam-

atory stimuli) and show that the Ag presentation properties were markedly impaired (29), perhaps suggesting that the mono-
ocytes generated under these conditions would have tolerogenic properties.

Prior experiments testing the influence of 1,25(OH)2D3 on mature (peripheral blood) NK function are varied and seemingly contradictory. In this study, to our knowledge we show for the first time that NK cells that develop from progenitors in the presence of 1,25(OH)2D3 show reduced differentiation and function. In con-
trast, we found that adult NK cells cultured with IL-15 were not affected from exogenous 1,25(OH)2D3 and showed no difference in expansion, cytokine production, or cytotoxicity. These findings are different from those previously published, including prior studies showing an inhibitory effect of 1,25(OH)2D3 on mature NK function (8, 30). However, caution is needed in interpreting these older experiments because they were performed using mixed cultures of PBLs following mitogen stimulation (31). The de-
scribed inhibitory effect of 1,25(OH)2D3 might be explained by the now known negative regulatory action of 1,25(OH)2D3 on IL-2 production by CD4+ T cells, especially because the investigators showed that exogenous IL-2 could overcome the inhibition (8, 30). Conversely, other investigators have shown that short-term culture (24 h) with 1,25(OH)2D3 activated NK function (15, 32). Vitamin D supplementation in patients undergoing chronic renal dialysis (33, 34) or with rickets (35) also showed improvements in NK function, suggesting that chronic deficiency may also negatively regulate NK function. NK cells are also known to exist in the uterus where they lack cytotoxicity but play a central role in embryo implantation (reviewed in Ref. 36). It is not clear how NK cells acquire their unique properties in the uterus, but it is interesting to note that vitamin D is produced by trophoblasts (37, 38) and that vitamin D deficiency is a risk factor for recurrent fetal loss after in vitro fertilization and embryo implantation (37, 39). Considering a dynamic cross-talk between NK cells and tropho-

FIGURE 6. Peripheral blood NK expansion and cytotoxicity with 1,25(OH)2D3. Cells were isolated from healthy donors, purified using CD3−CD56+ magnetic beads, and cultured for 7 d with 10 nm 1,25(OH)2D3 and analyzed for (A) total cell expansion (n = 4, p = 0.06) and (B) IFN-γ production in response to IL-12/IL-18 stimulation (n = 4, p = ns) and (C) cytotoxicity to K562 cells (n = 4, p = ns).
blasts perhaps suggests that 1,25(OH)2D3 in utero participates in the unique functions of this NK subset.

In the present study, using an in vitro NK cell differentiation system we show that 1,25(OH)2D3 has a negative regulatory effect on NK cell development from CD34+ progenitors, which is consistent with the literature showing an antiproliferative effect of this steroid hormone on numerous cell types. In addition, the NK cells that do differentiate in the presence of 1,25(OH)2D3 have attenuated inflammatory capacity, with reduced IFN-γ and cytotoxicity. Despite conditions that have been optimized for NK cell development, the addition of 1,25(OH)2D3 favors monocyte development. Collectively, these studies show that 1,25(OH)2D3 negatively influences NK differentiation from hematopoietic progenitors. An important caveat with these data is that both the activation of 25(OH)D3 to 1,25(OH)2D3 and the subsequent inactivation of 1,25(OH)2D3 are tightly regulated enzymatic processes that are difficult to model in vitro. In addition, the local concentrations of 1,25(OH)2D3 in the bone marrow or secondary lymphoid tissues (where NK cell development occurs) is not known. Future studies are needed to address whether vitamin D supplementation impacts NK cell number or function in experimental animals or human populations.

Disclosures
The authors have no financial conflicts of interest.

References