Regulation of *Mycobacterium*-Specific Mononuclear Cell Responses by 25-Hydroxyvitamin D$_3$

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**Abstract**

The active vitamin D metabolite, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), has been shown to be an important regulator of innate and adaptive immune function. In addition, synthesis of 1,25(OH)$_2$D$_3$ from 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) by the enzyme 1α-hydroxylase in monocytes upon activation by TLR signaling has been found to regulate innate immune responses of monocytes in an intracrine fashion. In this study we wanted to determine what cells expressed 1α-hydroxylase in stimulated peripheral blood mononuclear cell (PBMC) cultures and if conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ in PBMC cultures regulated antigen-specific immune responses. Initially, we found that stimulation of PBMCs from animals vaccinated with *Mycobacterium bovis* (M. bovis) BCG with purified protein derivative of M. bovis (M. bovis PPD) induced 1α-hydroxylase gene expression and that treatment with a physiological concentration of 25(OH)D$_3$ down-regulated IFN-γ and IL-17F gene expression. Next, we stimulated PBMCs from *M. bovis* BCG-vaccinated and non-vaccinated cattle with *M. bovis* PPD and sorted them by FACS according to surface markers for monocytes/macrophages (CD14), B cells (IgM), and T cells (CD3). Sorting the PBMCs revealed that 1α-hydroxylase expression was induced in the monocytes and B cells, but not in the T cells. Furthermore, treatment of stimulated PBMCs with 25(OH)D$_3$ down-regulated antigen-specific IFN-γ and IL-17F responses in the T cells, even though 1α-hydroxylase expression was not induced in the T cells. Based on evidence of no T cell 1α-hydroxylase we hypothesize that activated monocytes and B cells synthesize 1,25(OH)$_2$D$_3$ and that 1,25(OH)$_2$D$_3$ down-regulates antigen-specific expression of IFN-γ and IL-17F in T cells in a paracrine fashion.

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**Introduction**

Substantial evidence supports the notion that vitamin D insufficiency (serum 25(OH)D$_3$ concentrations <32 ng/mL or 80 nM) results in inadequate immune function and thus increased risk for infectious and autoimmune diseases [1]. For instance, an inverse correlation exists between serum 25(OH)D$_3$ and the risk for upper respiratory tract infections [2], tuberculosis [3,4], and multiple sclerosis [5,6]. Vitamin D supplementation also decreases the risk influenza A infection [7], decreases the relapse rate in multiple sclerosis patients [8], and enhances ex vivo immunity to *Mycobacteria tuberculosis* [9]. The actions of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$; the active hormone) on innate and adaptive immunity and the ability of immune cells to synthesize 1,25(OH)$_2$D$_3$ [10] provides further evidence for a link between vitamin D status and immune function. Understanding the mechanisms of vitamin D signaling in the immune system, consequently, provides critical insight for the vitamin D requirements of the immune system.

The vitamin D hormone has been known for some time to regulate key responses of innate and adaptive immunity. The actions of 1,25(OH)$_2$D$_3$ on the immune system occur through the vitamin D receptor (VDR). The VDR is present in most populations of immune cells [11,12,13] and controls the expression of genes that have promoters with accessible vitamin D response elements [14,15]. In human monocytes and macrophages, 1,25(OH)$_2$D$_3$ induces cathelicidin, CD14, defensin beta 4, and NOD2 gene expression [16,17,18,19]. In contrast to human monocytes, 1,25(OH)$_2$D$_3$ enhances inducible nitric oxide synthase (iNOS) and RANTES/CCL5 gene expression in bovine monocytes [20]. In regards to adaptive immunity, 1,25(OH)$_2$D$_3$ is a potent suppressor of lymphocyte proliferation and this has been observed for humans, cattle, and mice [21,22,23,24]. In addition, 1,25(OH)$_2$D$_3$ suppresses IFN-γ responses of T cells from humans, cattle and mice in vitro [25,26,27,28,29]. Recently, 1,25(OH)$_2$D$_3$ also was found to suppress IL-17A responses of human and mouse T cells [30,31]. In mouse models of autoimmune disease, 1,25(OH)$_2$D$_3$ suppresses Th1 and Th17-mediated inflammation.
Cathelicidin [32,33,34], and T cell VDR expression is required for 1,25(OH)2D3-mediated inhibition of experimental autoimmune encephalomyelitis (EAE) [35]. Altogether, in vitro and in vivo evidence show that 1,25(OH)2D3 acts on immune cells to regulate both innate and adaptive immunity, and that the actions of 1,25(OH)2D3 on adaptive immunity are similar among humans, cattle and mice.

The metabolism of 1,25(OH)2D3 is critical for immune function because of the potent effects of 1,25(OH)2D3 on innate and adaptive immunity. The enzyme that synthesizes 1,25(OH)2D3 from 25-hydroxyvitamin D3 (25(OH)D3) is 1α-hydroxylase (1α-OHase) [36]. In the vitamin D endocrine system, 1α-OHase is expressed in the kidney and is tightly regulated in response to calcium homeostasis via the parathyroid hormone in order to control the circulating concentration of 1,25(OH)2D3 [37]. However, the circulating concentration of 1,25(OH)2D3 does not affect vitamin D-mediated immune responses [38,39] and circulating 1,25(OH)2D3 does not increase when the immune system is activated [40]. Rather, monocytes and macrophages express 1α-OHase in response to toll-like receptor (TLR) signaling, and this has been shown for humans, cattle, and mice [20,41,42]. In addition, dendritic cells, B cells and T cells also have been found to express 1α-OHase to some degree upon activation [43,44]. However, 1α-OHase is predominantly upregulated in the CD14+ cells (monocytes/macrophages) from the inflamed mammary gland during mastitis in cattle [45]. Consequently, induction of 1α-OHase in immune cells enables regulation of 1,25(OH)2D3 concentration at sites of inflammation and this localized regulation is evident from animal models of inflammation. In cattle, the gene for 24-hydroxylase, the vitamin D catabolic enzyme that is highly upregulated by 1,25(OH)2D3, is expressed much higher in inflamed mammary tissue than in healthy tissue or circulating immune cells during mastitis [45]. Also in cattle, 1,25(OH)2D3 accumulated in granulomas during tuberculosis [46]. Finally, the concentration of 1,25(OH)2D3 increased in the spinal cords of mice during EAE, but did not change in serum [47]. Therefore, the immune system has a mechanism to control 1,25(OH)2D3 concentration locally independent of the endocrine system.

Subsequently, local control of 1,25(OH)2D3 metabolism by the immune system has been shown to have a significant impact on innate immunity [48]. For example, synthesis of 1,25(OH)2D3 by 1α-OHase in human monocytes induces their expression of cathelicidin [41]. Similarly, synthesis of 1,25(OH)2D3 by 1α-OHase in bovine monocytes enhances their expression of iNOS and RANTES [20]. So, 1,25(OH)2D3 produced in monocytes acts in an intracrine fashion to regulate vitamin-responsive genes.

As for adaptive immunity, monocyte production of 1,25(OH)2D3 has been suggested to also regulate T cell responses in a paracrine fashion [48]. However, lymphocytes also may be a source of 1,25(OH)2D3 and regulation of antigen-specific immune responses of T cells by conversion of 25(OH)D3 to 1,25(OH)2D3 in either monocytes or lymphocytes has yet to be shown. Therefore, the objectives of this study were to evaluate 1α-OHase gene expression in PBMC cultures in response to antigen stimulation and determine the effects of 25(OH)D3 on innate and adaptive immune responses in PBMC cultures.

To accomplish the objectives of this study we use PBMCs from calves vaccinated with Mycobacterium bovis bacilli Calmette-Guerin (M. bovis BCG), which elicits strong IFN-γ and IL-17 responses to purified protein derivative (PPD) of M. bovis [49]. The calf immune system has been found to serve as a good model of the human immune system for the study of tuberculosis and M. bovis BCG vaccination [50,51]. In addition, the concentration of 25(OH)D3 circulating in blood is similar between cattle and humans with typical concentrations ranging from 20 to 100 ng/mL in both species [32,33,34]. In cattle and humans symptoms of vitamin D toxicity are rarely observed with circulating 25(OH)D3 concentrations below 200 ng/mL [8,35,36]. Finally, as mentioned already, local control of 1,25(OH)2D3 synthesis by the immune system and 1,25(OH)2D3 regulation of T cell responses is similar between cattle and humans. Therefore, the outcome of this study will provide insight on the mechanisms of vitamin D signaling in the human and bovine immune systems.

Materials and Methods

Animals

Twelve male Holstein calves that were approximately 5 months to 12 months of age were used for this study. At 14 d of age, 8 calves were vaccinated subcutaneously in the midcervical region with 10⁷ cfu of M. bovis BCG (Pasteur strain). M. bovis BCG was prepared for vaccination as previously described [57]. The remaining 4 calves were not vaccinated. The NADC animal care and use committee approved the care and treatment of animals used in this study (Animal Protocol #3982).

Peripheral blood mononuclear cell cultures

Blood from the jugular vein was collected in 2× acid citrate dextrose. Blood was centrifuged and buffy coats were collected. Contaminating RBCs were removed by hypotonic lysis. PBMCs were isolated by density gradient centrifugation. PBMC were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA). For gene expression assays, PBMCs were cultured at a concentration of 1.5×10⁷ cells/ml in 96-well (200 μl/well) or 6-well (2 ml/well) tissue culture plates for 24 h at 37°C in 5% CO2. For determination of nitric oxide and IFN-γ production, PBMCs were cultured at 1×10⁶ cells/ml in a 96-well plate (200 μl/well) for 24, 48, and 72 h.

LPS from Serratia marcescens (Sigma-Aldrich), pokeweed mitogen (PWM; Sigma-Aldrich) and purified protein derivative from M. bovis (M. bovis PPD) (Prionics, Zurich, Switzerland) were added at 100 ng/ml, 5 μg/ml, and 10 μg/ml, respectively, to PBMC cultures. The vitamin D metabolites, 25(OH)D3 and 1,25(OH)2D3 (Sigma-Aldrich) were diluted in 100% ethanol and added to fetal bovine serum (FBS; Hyclone, Waltham, MA) at 10× the final desired concentration. The concentrations of 25(OH)D3 and 1,25(OH)2D3 in ethanol were confirmed by UV spectroscopy. FBS with ethanol (vehicle) or the vitamin D metabolites was added to PBMC cultures to a final concentration of 10% FBS. The final concentration of ethanol did not exceed 0.04%.

Cell sorting

PBMCs from 7 BCG-vaccinated and 4 non-vaccinated calves were cultured with 10 μg/ml M. bovis PPD and 0 or 100 ng/ml 25(OH)D3 for 24 h in 6 well plates. Cells were removed from the wells with cold PBS and scraping. Cells were labeled with anti-CD14 (CAM36A; mouse IgG1), anti-ÎµM (PIG45A; mouse IgG2b), or a cocktail of anti-CD3 (MM1A; mouse IgG1), anti-CD4 (CACT83B; mouse IgG1), anti-CD8 (MAQ111A; mouse IgG2b), or a cocktail of anti-CD3 (MM1A; mouse IgG1), anti-CD4 (CACT83B; mouse IgM), anti-CD8 (MAQ111A; mouse IgG2b) and use committee approved the care and treatment of animals used in this study (Animal Protocol #3982).
was measured using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were converted to micromoles per liter using a standard curve that was generated by addition of 0 to 100 µM sodium nitrite to fresh culture media.

**Measurement of IFN-γ production**

The concentration of IFN-γ in PBMC culture supernatants was determined by an ELISA using the Endogen Bovine IFNγ Screening Set (Pierce Biotechnology, Rockford IL) according to the manufacturer’s instructions. The absorbance at 450 nm minus the absorbance at 550 nm was measured with the FlexStation 3 plate reader and the values were converted to picograms per milliliter by using a standard curve.

**Statistical Analysis**

Analysis of variance was performed using PROC GLM of SAS (SAS Institute Inc., Cary, NC). The model accounted for effects of treatment, cell type, and calf or vaccination status. ΔΔCt values were used in the analyses of gene expression. The average ΔΔCt values ± SE were transformed using the equation $2^{-\Delta\Delta C_{t}}$. The expression of each gene is presented as the mean fold increase ± SE relative to non-stimulated controls. Multiple comparison tests of the means were made using the Tukey adjustment.

**Results**

*M. bovis* PPD-activation of vitamin D signaling in PBMCs

By stimulating PBMCs from *M. bovis*-BCG-vaccinated calves with LPS, PWM, or *M. bovis* PPD, we found that 1α,25(OH)2D3 was found to suppress IFN-γ production (Fig. 1E–G). We also measured iNOS, RANTES, IFN-γ, IL-17A, and IL-17F gene expression. Neither iNOS nor RANTES was affected by *M. bovis* PPD or LPS stimulation, but RANTES was upregulated by PWM stimulation (Fig. 1C and D). IFN-γ, IL-17A, and IL-17F were upregulated in PBMCs stimulated with PWM or *M. bovis* PPD, however, they were not affected by LPS stimulation (Fig. 1E–G).

Previously, treatment of *M. bovis* PPD-stimulated PBMCs with exogenous 1,25(OH)2D3 was found to suppress IFN-γ production [29]. And recently, we showed that conversion of 25(OH)2D3 to 1α,25(OH)2D3 by 1α,25(OH)ase in activated bovine monocytes up-

### Table 1. Primer sequences for real-time PCR.

| Gene (alternate name) | Accession no. | Strand | Sequence (5′ - 3′) |
|-----------------------|---------------|--------|--------------------|
| 1α,OHase (CYP27B1)$^2$ | NM_001192284 | Forward | TGGGACACAGATTTCTGATCGTC | TTCTCAGACTGGTGTCCTGATGCT |
| 24α-HOase (CYP24A1)$^2$ | NM_001191417 | Forward | GAAGACTGGCAGAGGCTGAG | CAGCCCAAGACCTCGTTGATT |
| IFN-γ | NM_174086 | Forward | GATTCAAATTCCGGTGGATG | GCAGGAGGACCATTACGTTG |
| IL-17A | NM_001101152 | Reverse | GTGAGGTCTGGAGGGTCAAA | GGGCATTACCTTCGAACAGA |
| RANTES (CCLS)$^2$ | NM_001008412 | Forward | TCCATCTCAGACGGAACCATAG | AAAAGACACCTCAGGAGG |
| RPS9 | NM_001167932 | Forward | AGCCACCGCTTCCATTTCA | AACAAGCCGCTTCCGTCAT |

1Accession numbers for mRNA sequences from NCBI database.
2Primer sequences have been published previously [20].

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regulated iNOS and RANTES expression [20]. Because 1α-OHase gene expression was upregulated in *M. bovis* PPD-stimulated PBMCs, we wanted to determine the effect of 25(OH)D3 on gene expression in *M. bovis* PPD-stimulated PBMCs.

Addition of 100 ng/mL 25(OH)D3, a physiological concentration [54], to resting PBMCs did not affect expression of any of the genes tested (Fig. 2). Addition of 4 ng/mL 1,25(OH)2D3, a concentration 2 to 3 orders of magnitude greater than normal serum 1,25(OH)2D3, did upregulate 24-OHase and RANTES gene expression in resting PBMCs (*P*<0.05; Fig. 2A and C). Stimulation of PBMCs with *M. bovis* PPD suppressed 24-OHase gene expression (*P*<0.05; Fig. 2A), which is consistent with LPS stimulation of bovine monocytes [20]. However, addition of either 25(OH)D3 or 1,25(OH)2D3 to stimulated PBMCs increased 24-OHase gene expression relative to PBMCs that were stimulated with *M. bovis* PPD alone. Similarly, iNOS and RANTES were upregulated in *M. bovis* PPD-stimulated PBMCs treated with 25(OH)D3 or 1,25(OH)2D3 compared to PBMCs that were stimulated with *M. bovis* PPD alone (*P*<0.05; Fig. 2B and C). IL-17F gene expression was decreased in stimulated PBMCs that were treated with 1,25(OH)2D3 (*P*<0.05; Fig. 2F). IFN-γ, IL-17A, and IL-17F gene expression was decreased by 25(OH)D3 treatment, but the decrease was not statistically significant (*P*>0.05; Fig. 2D–F).

**Figure 1.** 1α-hydroxylase (1α-OHase) gene expression in PBMC cultures. PBMC cultures from eight *M. bovis*-BCG-vaccinated calves were treated with 100 ng/ml LPS, 5 μg/ml PWM, or 10 μg/ml of *M. bovis* PPD or received no treatment (CTRL) as indicated for 24 h. The amount of 1α-OHase (A), VDR (B), iNOS (C), RANTES (D), IFN-γ (E), IL-17A (F), and IL-17F (G) mRNA was determined by quantitative real-time RT-PCR and was normalized to the amount of RPS9 mRNA in each sample. Expression of each gene is relative to non-stimulated cultures. Error bars represent SE. *** Mean is different from non-stimulated PBMC; *P*<0.001.
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In addition to gene expression, we measured nitric oxide and IFN-γ production by *M. bovis* PPD-stimulated PBMCs treated with graded doses of 25(OH)D$_3$ form 0 to 125 ng/mL (Fig. 3). Like iNOS gene expression, 100 ng/mL 25(OH)D$_3$ increased nitric oxide production, as measured by nitrite in the culture supernatant, after 40 and 72 h in culture (Fig. 3A). Furthermore, treatment with 25 to 125 ng/mL 25(OH)D$_3$ increased nitric oxide production by the stimulated PBMCs in a dose dependent manner. Addition of 25(OH)D$_3$ suppressed IFN-γ production in *M. bovis* PPD-stimulated cultures after 24, 48, and 72 h in culture (P<0.05; Fig. 3B), but the effect of 25(OH)D$_3$ did not occur in linear fashion.

**Cell type-specific expression of 1α-OHase and VDR**

Several cell types have been reported to express 1α-OHase, including activated monocytes, T cells, and B cells [41,43,44]. We sorted PBMCs that had been stimulated with *M. bovis* PPD from BCG-vaccinated animals according to surface expression of CD3, IgM, and CD14 by using FACS (Fig. 4) to determine what populations of cells in PBMCs were expressing 1α-OHase upon activation. By sorting the stimulated PBMCs, we found that 1α-OHase was predominantly expressed in the CD14$^+$ population of cells (P<0.001; Fig. 4A). 1α-OHase expression was also induced in IgM$^+$ cells from vaccinated calves (P<0.001). Relative to 1α-OHase expression in non-stimulated, non-sorted PBMCs, the expression of 1α-OHase did not increase in the CD3$^+$ cells isolated from the stimulated PBMC cultures (Fig. 4A). Unlike 1α-OHase, VDR gene expression did not differ significantly between cell types in PBMC cultures from vaccinated calves, but IgM$^+$ cells from 25(OH)D$_3$ treated cultures did have somewhat lower VDR expression (Fig. 4B).

**Cell type-specific effects of 25(OH)D$_3$ on gene expression**

We also compared gene expression in cells from *M. bovis* PPD-stimulated PBMCs that were treated with 100 ng/ml 25(OH)D$_3$ with cells from *M. bovis* PPD-stimulated PBMCs that were not treated with 25(OH)D$_3$. Treatment with 25(OH)D$_3$ increased 24-OHase, iNOS, and RANTES gene expression in both CD14$^+$ cells and IgM$^+$ cells from the BCG-vaccinated calves (P<0.05; Fig. 4C–E). In contrast, 25(OH)D$_3$ treatment decreased expression of IFN-γ by over 60% and IL-17F by nearly 50% in the CD3$^+$ cells from the BCG-vaccinated calves (P<0.05; Fig. 4F and H). IL-17A expression in the CD3$^+$ cells was also down-regulated by 25(OH)D$_3$ treatment, but to a lesser extent (P>0.05; Fig. 4G).

**Comparison of responses between BCG-vaccinated and non-vaccinated animals**

Finally, we compared changes in gene expression caused by *M. bovis* PPD stimulation and 25(OH)D$_3$ in cells from non-vaccinated animals to the changes observed in cells from BCG-vaccinated animals. In PBMCs from non-vaccinated animals, 1α-OHase was induced in CD14$^+$ cells by *M. bovis* PPD stimulation like in CD14$^+$ cells from BCG-vaccinated animals (Fig. 4A). Unlike the PBMCs from BCG-vaccinated calves, 1α-OHase was not detected in IgM$^+$ cells from non-vaccinated calves.

![Figure 2. Effects of 1,25(OH)2D3 and 25(OH)D3 on gene expression in PBMC cultures.](https://www.plosone.org/doi/fig/10.1371/journal.pone.0021674.g002)
from the non-vaccinated animals (Fig. 4B). VDR expression in CD3+ and CD14+ cells was similar between vaccinated and non-vaccinated calves. Neither 24-OHase, iNOS, or RANTES expression was affected by 25(OH)D3 in CD14+ cells and IgM+ cells from the non-vaccinated animals like it was in the BCG-vaccinated animals (Fig. 4C–E). Finally, IFN-γ, IL-17A, and IL-17F were not induced by M. bovis PPD stimulation in the CD3+ cells from non-vaccinated animals as they were in CD3+ cells from BCG-vaccinated animals (Fig. 4F–H).

**Discussion**

For over two decades now, 1,25(OH)2D3 has been known as an important regulator of adaptive immunity, suppressing lymphocyte proliferation and IFN-γ production [28,60]. The implications of 1,25(OH)2D3 on adaptive immunity are further realized in animal models of T cell-mediated autoimmunity as 1,25(OH)2D3 inhibits disease progression [32,34,61,62]. Recently, 1,25(OH)2D3 also was found to be an important regulator of innate immunity by enhancing antimicrobial properties of macrophages [16,18]. Vitamin D-mediated immune responses, however, do not correlate with the circulating concentration of 1,25(OH)2D3 [38,39]. Therefore, local synthesis of 1,25(OH)2D3 is a critical factor in regulating both innate and adaptive immunity. Previously, induction of 1α-OHase expression in macrophages was shown to occur upon activation by TLR 2/1 or TLR 4 signaling and enable them to convert 25(OH)D3 to 1,25(OH)2D3 [20,41,63]. Synthesis of 1,25(OH)2D3 in macrophages, in turn, enhanced their innate antimicrobial properties in an intracrine fashion [48]. In this study, we give evidence that endogenous synthesis of 1,25(OH)2D3 also occurs in antigen-stimulated PBMC cultures and regulates key aspects of adaptive immunity.

In this study we found that 1α-OHase gene expression was induced in CD14+ cells (monocytes/macrophages) and IgM+ (B cells), but not in CD3+ (T cells) cells in M. bovis PPD-stimulated PBMC cultures. Furthermore, treatment of M. bovis PPD-stimulated PBMC cultures with 25(OH)D3 enhanced iNOS and RANTES expression in monocytes and B cells and suppressed antigen-specific IFN-γ and IL-17F responses in T cells. Based on this evidence, we hypothesize that 1,25(OH)2D3 was produced in monocytes and B cells acted on monocytes and B cells in an intracrine fashion to upregulate iNOS and RANTES expression and on T cells in a paracrine fashion to suppress M. bovis PPD-specific IFN-γ and IL-17F responses (Fig. 5).

M. bovis PPD is a crude extract and as such likely contains antigens that activate both the innate and adaptive immune systems. Therefore, in PBMC cultures innate antigen presenting cells (e.g., monocytes) recognize TLR ligands, such as lipoproteins, become activated and then express 1α-OHase. The APCs also internalize protein from the M. bovis PPD, process it and then present it on their surface as peptide associated with MHC. Activation of T cells specific for M. bovis PPD is then caused by the
interaction of the specific T cell receptor (TCR) with its cognate MHC/antigen. Likewise, B cells recognize antigen through IgM on their surfaces, and along with co-stimulation from T cells, become activated and express 1α-OHase. We suggest that production of 1,25(OH)₂D₃ by 1α-OHase in activated monocytes and B cells can alter the IFN-γ and IL-17F responses that are the result of the TCR/MHC/antigen interaction between T cells and APCs.

There are multiple possibilities as to how 1,25(OH)₂D₃ suppressed IFN-γ and IL-17F gene expression in T cells. VDR expression in the T cells was similar to that in monocytes in this study and purified T cells do respond to 1,25(OH)₂D₃ [25,64]. Also, T cell VDR expression is required for 1,25(OH)₂D₃-mediated inhibition of experimental autoimmune encephalomyelitis in mice [35]. Therefore, the T cells in the PBMC cultures likely had the ability to respond to 1,25(OH)₂D₃ secreted from the monocytes and B cells. Consequently, activation of the VDR in T cells could have directly suppressed IFN-γ and IL-17F expression. However, 1,25(OH)₂D₃ failed to suppress IFN-γ production in fully differentiated Th1 cells [61]; so, 1,25(OH)₂D₃ may have regulated genes in T cells that influenced T cell differentiation or sensitized them to apoptosis. Alternatively, up-regulation of nitric oxide production by 1,25(OH)₂D₃ in monocytes and B cells could have induced apoptosis in the surrounding T cells and resulted in suppressed IFN-γ and IL-17F expression. A combination of several mechanisms also is possible and we have not ruled out the

Figure 4. Cell-type specific regulation of vitamin D signaling. PBMCs from seven M. bovis-BCG-vaccinated calves (black bars) and four non-vaccinated calves (grey bars) were treated with 10 μg/ml of M. bovis PPD and 0 or 100 ng/ml 25(OH)D₃ as indicated for 24 h. After treatment, PBMCs were sorted by FACS according to CD3/4/8/iTCR (CD3; T cell), IgM (B cell), and CD14 (monocyte) expression on the cell surface. The amount of 1α-OHase (A), VDR (B), 24-OHase (C), iNOS (D), RANTES (E), IFN-γ (F), IL-17A (G), and IL-17F (H) mRNA in the sorted cells was determined by quantitative real-time RT-PCR. The amount of mRNA for each gene was normalized to the amount of RPS9 mRNA in each sample. Expression of each gene in each cell type is relative to non-stimulated, non-sorted PBMCs. Error bars represent SE. * P<0.05, *** P<0.001, Means are different.

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possibility that T cells are able to synthesize their own 1,25(OH)2D3. Therefore, further experiments are needed to determine if regulation of T cell responses by 1,25(OH)2D3 strictly depends on synthesis of 1,25(OH)2D3 in monocytes or B cells and how 1,25(OH)2D3 is regulating T cell responses.

In any case, treatment of antigen-stimulated PBMCs with 25(OH)D3 suppressed antigen-specific IFN-γ and IL-17F expression in T cells, which indicates that synthesis of 1,25(OH)2D3 by immune cells has significant implications in regulating adaptive immunity. IFN-γ is a potent activator of macrophages and is mainly produced by Th1 cells [65]. Th1-mediated responses are critical in the defense against intracellular infections, such as tuberculosis [66,67]. IL-17A and IL-17F are produced by Th17 cells and play major roles in neutrophil recruitment and protection against intracellular and extracellular bacterial infections [68,69,70]. Self reactive Th1 and Th17 cells, however, are involved in the development of autoimmune disorders [71] and inhibition of animal models of autoimmunity by 1,25(OH)2D3 is thought to occur, in part, by suppression of self-reactive Th1 and Th17 cells [72]. Although suppression of Th1 and Th17 responses to bacterial antigens by 1,25(OH)2D3 would seem to attenuate the immune response against bacterial infections, keep in mind that 1,25(OH)2D3 also enhances the antimicrobial activity of macrophages [41]. So overall, production of 1,25(OH)2D3 by immune cells serves to limit inflammation caused by Th1 and Th17 effector cells, but ultimately improves defense against bacterial infections by boosting the innate antimicrobial response.

In addition to suppression of T cell responses by 1,25(OH)2D3 synthesis in PBMC cultures, treatment of PBMCs with 25(OH)D3 upregulated antigen-specific B cell iNOS and RANTES expression. We had previously shown that monocyte iNOS and RANTES expression depends on availability of 25(OH)D3 [20], but not B cell iNOS and RANTES. Nitric oxide produced by iNOS in macrophages is considered to be an antimicrobial molecule. However, nitric oxide produced by the monocytes and B cells may suppress proliferation of T cells [73]. So, as mentioned above, 1,25(OH)2D3 may suppress T cell responses in part by enhancing B cell and monocyte iNOS expression. RANTES is a
chemokine originally found to be expressed by T cells [74], but also has been found to be expressed by alveolar macrophages in cattle [75]. We speculate that upregulation of RANTES in monocytes and B cells by 1,25(OH)2D3 would enhance recruitment of immune cells to the site of inflammation, but the implications of 1,25(OH)2D3—upregulation of RANTES in monocytes and B cells will need to be investigated.

Finally, the ability of 1α-OHase in monocytes and B cells to synthesize 1,25(OH)2D3, and subsequently regulate 1,25(OH)2D3-mediated immune responses, depends on the availability of 25(OH)D3. The circulating concentration of 25(OH)D is primarily mediated by dietary intake of vitamin D3 and sun exposure [76]. Current recommendations for vitamin D in humans and cattle target a circulating concentration of 25(OH)D of 20 to 50 ng/ml [57,57]. However, 25(OH)D concentrations above 30 ng/ml may be necessary for optimal immune function [1]. In addition, vitamin D insufficiency (serum 25(OH)D below 20 ng/ml) is widespread, vitamin D insufficiency (serum 25(OH)D below 30 ng/ml) and even deficiency (serum 25(OH)D below 20 ng/ml) are necessary for optimal immune function [1]. In addition, vitamin D insufficiency (serum 25(OH)D below 30 ng/ml) and even deficiency (serum 25(OH)D below 20 ng/ml) are widespread, indicating that current recommendations for vitamin D intake may be inadequate [78,79]. Previously, and here we have shown that 1,25(OH)2D3—regulated innate immune responses increase linearly from 0 to 125 ng/ml of 25(OH)D3 [20]. This observation leads to the question of what concentration is necessary for optimal immune functionality if below 30 ng/ml is insufficient? Based on the requirement of 25(OH)D3 by the immune system for signaling mechanisms and evidence from epidemiological studies, vitamin D requirements need to be re-evaluated to ensure proper immune function.

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Author Contributions

Conceived and designed the experiments: CN BN TR WW DB JL. Performed the experiments: CN. Analyzed the data: CN BN TR WW DB JL. Contributed reagents/materials/analysis tools: CN BN TR WW JL. Wrote the paper: CN TR JL.

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