Effect of in vitro and in vivo 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens during a coccidia challenge

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ABSTRACT: This article describes the in vitro and in vivo effects of a 25-hydroxycholecalciferol (25(OH)D) treatment in layer hens during a mixed coccidia challenge. HD11 cells (chicken macrophage cell line) were treated in vitro with a coccidia antigen or in a medium supplemented with either 1,25-dihydroxycholecalciferol (1,25(OH)2D) or 25(OH)D. HD11 cells treated in vitro with 200 nM of 1,25(OH)2D had increased nitrite production (P < 0.01) compared with HD11 cells treated with 0 or 200 nM of 25(OH)D. Treating HD11 cells with 25(OH)D decreased IL-10 mRNA by 1.7-fold, but 1,25(OH)2D treatment increased the amount of IL-10 mRNA by 2.7-fold (P < 0.01) compared with the group treated with 0 nM of 25(OH)D. Post-coccidial antigen stimulation, 25(OH)D or 1,25(OH)2D treatment decreased (P < 0.01) 1α-hydroxylase mRNA amounts in HD11 cells. Stimulating primary T cells in vitro with Concanavalin A (Con-A) decreased (P = 0.020) the 1α-hydroxylase mRNA amounts by 3-fold. ConA-B1-VICK cells (chicken T cell line) stimulated with 100 nM 1,25(OH)2D or with supernatants from HD11 cells treated with 25(OH)D plus lipopolysaccharide (LPS) had 1.3-fold less (P < 0.01) interferon (IFN)-γ mRNA compared with the group treated with 25(OH)D. Layer birds were fed a basal diet supplemented with 25(OH)D at 6.25, 25, 50, or 100 μg/kg, and at 21 d of age orally challenged with 1 × 10^5 live coccidia oocysts. Compared with birds fed similar levels of 25(OH)D and unchallenged with the coccidia oocyst, birds challenged with the coccidia oocyst had 15% reduced BW gain in the groups supplemented with either 6.25, 25, or 50 μg/kg of 25(OH)D, but only a 4% reduced BW gain in birds fed 100 μg/kg of 25(OH)D (P < 0.01). Birds fed 100 μg/kg 25(OH)D had decreased (P = 0.012) CD8+ cell percentages in cecal tonsils in both coccidial oocyst challenged and unchallenged birds, compared with birds fed 6.25 μg/kg 25(OH) and unchallenged with coccidial oocysts. At 15 d post-coccidia challenge, birds fed 100 μg/kg 25(OH)D and challenged with coccidial oocysts had 17% more CD4+CD25+ cells (P = 0.018) in the cecal tonsils compared with the birds fed 25(OH) and unchallenged with coccidial oocysts. At d 6 post-coccidia challenge, birds fed 100 μg/kg 25(OH)D had a 3.5-fold increase (P < 0.01) in IL-10 mRNA amounts in the cecal tonsils compared with birds fed 6.25 μg/kg 25(OH)D. In conclusion, supplementing birds with 100 μg/kg 25(OH)D could be a nutritional strategy to reduce the production losses post-coccidia challenge.

Key words: 25-hydroxyvitamin D, coccidiosis, HD11 cells, layer chicken, T cells
Vitamin D is an immunomodulatory nutrient, and the active form of vitamin D, 1,25-dihydroxycholecalciferol (1,25(OH)2D), improves host defenses against intracellular pathogens like Mycobacterium tuberculosis by increasing macrophage antimicrobial peptide cathelicidin (Sato et al., 2013). Earlier we identified that in vitro supplementation of 25-hydroxyvitamin D (25(OH)D), an inactive form of vitamin D, increases macrophage nitric oxide (NO) production during an inflammatory challenge (Morris and Selvaraj, 2014). Replication of Eimeria tenella is inhibited by NO (Lee et al., 2011; Lillehoj and Li, 2004). Active vitamin D is produced from 25(OH)D by the enzyme 1α-hydroxylase, expressed mostly in kidneys (Nykjaer et al., 1999). However, extra-renal presence of 1α-hydroxylase in macrophages facilitates local production of active vitamin D from 25(OH)D (Morris and Selvaraj, 2014). Of interest is identifying whether supplementing 25(OH)D improves host defense against coccidiosis.

Because mammalian T cells have limited expression of the 1α-hydroxylase enzyme, they rely on extrinsic sources of 1,25(OH)2D (Jeffery et al., 2012) for their conversion into a regulatory phenotype that suppresses excess inflammatory responses (Jeffery et al., 2009). Identifying whether chicken T cells can convert 25(OH)D into 1,25(OH)2D during a coccidial infection will promote application of 25(OH)D to prevent excess inflammatory response associated with coccidiosis. This study investigated the in vitro and in vivo effects of 25(OH)D supplementation during a coccidia infection in chickens.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University, Ohio. Specific pathogen-free layer birds (White Leghorn) were housed in individual battery cages (40 [long] by 27 [wide] by 12 [high] inches) and provided feed and water ad libitum.

Preparation of Coccidial Antigens for In Vitro Studies

Coccidial antigens for in vitro studies were prepared by slight modifications to the established procedures (Annamalai and Selvaraj, 2012; You, 2014). Briefly, 1 × 107 coccidial oocysts containing a mixture of Eimeria acervulina, Eimeria maxima, and Eimeria tenella (Inovocox; Zoetis, Florham Park, NJ) were resuspended in 6 mL of 1× PBS and placed in a −70°C freezer for 30 min to begin freeze–thaw cycles. Following freezing, coccidial oocysts were subsequently thawed at room temperature for 45 min. The freeze–thaw cycle was repeated for a total of 3 times. At the end of the final freeze–thaw cycle, 500 μL of oocysts were mixed with approximately an equal volume of 0.4- to 0.6-mm glass beads and subjected to a 4-min oscillation cycle of 50 oscillations/s in a TissueLyser LT (Qiagen, Valencia, CA). The oscillation cycles were repeated 4 times with a resting period of 10 to 15 s between each cycle. The lysed solution was centrifuged at 9,000 × g for 6 min to remove intact cells and cell debris. The protein content of the supernatant was determined using the Nanodrop Spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE).

Effect of 25(OH)D Treatment on HD11 Cell Nitrite Production

HD11 cells were obtained from Mike Kogut, Southern Plains Agricultural Research Center, TX. HD11 cells, a chicken macrophage cell line, were grown in 48-well plates (4 × 105 cells/well) in 500 μL of Roswell Park Memorial Institute (RPMI)-1640 growth medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential AA, L-glutamine, and 2% penicillin and streptomycin and allowed to adhere overnight at 37°C and 5% CO2. Following overnight incubation, the medium was removed, and fresh RPMI medium, supplemented with 200 nM of 25(OH)D (Santa Cruz Biotechnology, Dallas, TX) or 1,25(OH)2D (Cayman Chemical, Seattle, WA), was added in 8 replications (n = 8) and stimulated with 15 μg/well coccidial antigens for 12 h. Nitrite content in the cell culture supernatant was quantified using Griess reagent (Ricca Chemical Company, Arlington, TX) following the manufacturer’s instructions (Annamalai and Selvaraj, 2012).

Effect of 25(OH)D Treatment on IL-1β, IL-10, and 1α-hydroxylase mRNA Content of HD11 Cells

HD11 cells (4 × 105 cells/well) were grown in 48-well plates in 500 μL of RPMI medium and allowed to adhere overnight at 37°C and 5% CO2. Following overnight incubation, medium was removed and fresh RPMI medium supplemented with 200 nM of 25(OH)D or 1,25(OH)2D was added in 8 replications (n = 8). HD11 cells were stimulated with 15 μg/well coccidial antigen (control) or with coccidial antigen in the presence of 200 nM of 25(OH)D or 1,25(OH)2D for 12 or 48 h, respectively. At both 12 and 48 h, the cell culture supernatant was centrifuged at 9,000 × g, and 1000 μL of supernatant were reverse transcribed into cDNA. cDNA was analyzed for relative amounts of IL-1β (5’-tctcctcgacgacaatgtg-3’ and 5’-cagccgtagaagagagc-3’), IL-10 (5’-caatcagggaagagc-3’ and 5’-gggaggaccaacctgctgta-3’), and 1α-hydroxylase (5’-ttgctgctgcaagactg-3’).
and 5'-actgccccacctcttggtt-3') mRNA amounts by quantitative real-time PCR (iCyler; Bio-Rad, Hercules, CA) using SyBr green after normalizing for β-actin (5'-aagctgagcttcaaccac-3' and 5'-gactgctgctgacactctc-3') mRNA amounts as described previously (Shanmugasundaram and Selvaraj, 2010, 2011). Annealing temperatures for IL-1β, 1α-hydroxylase, and IL-10 primers were 57.5, 55, and 58°C, respectively (Morris and Selvaraj, 2014). The fold change from the reference was calculated as 2^((Ct Sample)−(Ct Reference)) where Ct is the threshold cycle (Selvaraj et al., 2010). The reference group was the control group stimulated with 15 µg/well of coccidial antigen. The threshold (Ct) values were determined by iQ5 software (Bio-Rad). Because insufficient RNA was obtained for some samples at 48 h, the replication size was 6 (n = 6) for real-time-PCR analysis.

**Isolation of Primary T cells and Estimating 1α-hydroxylase mRNA Content**

The thymus was isolated from 8- to 10-wk-old White Leghorn chickens, and mononuclear cells were obtained using Ficoll Density Gradient Separation (Histopaque 1077; Sigma, St. Louis, MO) by methods described earlier (Shanmugasundaram and Selvaraj, 2013). CD4+ T cells were isolated using MACS Microbeads technology (Miltenyi Biotec, Auburn, CA) by positive selection using mouse anti-chicken CD4-IgG-coated with magnetic beads (Shanmugasundaram and Selvaraj, 2012a). Purity of isolated CD4+ cells was determined by a flow cytometer (Guava easyCyte; Millipore, Billerica, MA) and was found to be over 90%. Isolated CD4+ cells (1 × 10^6) were grown in an RPMI medium supplemented with 2.5% FBS, 2% chicken serum, and 2% penicillin plus streptomycin and stimulated with 1 µg/mL of Concanavalin A (Con-A; Sigma Aldrich) for 48 h at 37°C. At the end of stimulation, RNA was extracted from CD4+ cells as described earlier, reverse transcribed into cDNA, and analyzed for relative amounts of 1α-hydroxylase mRNA amounts by quantitative real-time PCR after normalizing for β-actin.

**Effect of 25(OH)D Treatment on a Chicken T Cell Line (ConA-B1-VICK) on Interferon-γ mRNA Content**

RPMI medium supplemented with 2.5% FBS, 2% chicken serum, and 2% penicillin plus streptomycin was used for growing ConA-B1-VICK cells (ATCC, Manassas, VA). ConA-B1-VICK cells (2 × 10^7 cells/well) were stimulated with 7.5 µg/mL of Con-A for 120 h at 37°C and 5% CO2 in 48-well plates in an RPMI medium containing either 200 nM of 25(OH)D, 100 nM 1,25(OH)2D, or with a 1:5 dilution of supernatants from HD11 cells treated with 25(OH)D in the presence or absence of an lipopolysaccharides (LPS) stimulation. At the end of stimulation, RNA was extracted from ConA-B1-VICK cells as described earlier, reverse transcribed into cDNA, and analyzed for relative amounts of 1α-hydroxylase and interferon (IFN)-γ mRNA (5'-gtgagaggtgaagatcatga-3' and 5'-gtgttcgtgtgtgtct-3') amounts by quantitative real-time PCR after normalizing for β-actin as described above (Morris et al., 2014; Shanmugasundaram and Selvaraj, 2013). Annealing temperature for IFN-γ was 55°C. The reference group was the group stimulated with 7.5 µg/mL of Con-A in a RPMI media containing 200 nM of 25(OH)D.

**Animals, Housing, and Coccidial Infection**

A total of 200 1-d-old White Leghorn chicks hatched at the Ohio Agricultural Research and Development Center (Wooster, Ohio) hatchery were randomly distributed to 1 of 4 treatment groups. Each treatment was replicated in 10 individual battery cages (n = 10) with a total of 5 chicks in each individual battery cage. All birds were wing tagged and weighed individually. The 4 experimental groups were fed a basal diet (Table 1) supplemented with 25(OH)D (HyD; DSM Nutritional Products, Heerlen, Netherlands) at doses of 6.25, 25, 50, or 100 µg/kg, which are equivalent to 250, 1,000, 2,000, or 4,000 IU of cholecalciferol. The mean BW of chicks at d 1 in the different experimental groups supplemented with 25(OH)D at doses of 6.25, 25, 50, or 100 µg/kg were 41.5, 42, 42.1, and 41 g, respectively. At 21 d of age birds were weighed. Five individual battery cages per treatment (n = 5) were chosen, and all birds in the pen were orally challenged with 1 × 10^5 live coccidial oocysts (Inovocox; Zoetis) in 100 µL of PBS, as described previously, to induce a coccidial infection (Annamalai and Selvaraj, 2012). The birds in the remaining 5 battery cages/treatment were left unchallenged. The experimental design was a 4 × 2 factorial arrangement of treatments, and the unchallenged birds in each treatment group served as the control.

**Effect of Different Doses of 25(OH)D on BW Gain and Fecal Coccidial Oocyst Load Post-Coccidia Challenge**

Birds were weighed individually on the day of coccidial challenge and on d 6 post-coccidia challenge. The gain in BW over a 6-d period was calculated as the difference in the BW compared with the initial BW and expressed as a percentage. On d 6 and 15, post-coccidia challenge fecal samples were collected from individual battery cages in air-tight plastic bags and stored at 4°C.
until further analysis. On the day of analysis, samples were homogenized, and coccidial oocysts were enriched using a salt flotation technique described previously (Levine et al., 1960). Coccidial oocysts were diluted and counted using the McMaster Counting Chamber (Chalex Corporation, Ketchum, ID).

Effect of Different Doses of 25(OH)D Treatment on CD4\(^+\), CD8\(^+\), and CD4\(^+\)CD25\(^+\) Cell Percentage in Cecal Tonsils Post-Coccidia Challenge

One bird was selected randomly from each individual battery cage \((n = 5)\) on d 14 and 15 post-coccidia challenge. Cecal tonsils were collected to determine the percentage of CD4\(^+\) and CD8\(^+\) cells on d 14 and of Tregs (CD4\(^+\)CD25\(^+\)) on d 15. Single cell suspensions of the cecal tonsils were enriched for the mononuclear cells by density gradient separation as described previously (Shanmugasundaram and Selvaraj, 2012b). The percentages of CD4\(^+\), CD8\(^+\), and Tregs (CD4\(^+\)CD25\(^+\)) in the mononuclear cell populations were determined in a flow cytometer as described previously (Shanmugasundaram and Selvaraj, 2012b). CD4\(^+\)-CD25\(^+\) cell percentages were expressed as a percentage of total CD4\(^+\) cells to facilitate comparison between samples.

Effect of Different Doses of 25(OH)D Treatment on IL-1β, IL-10, and 1α-hydroxylase mRNA Amounts in Cecal Tonsils and Liver

At 6 d post-coccidia challenge, 1 bird was selected randomly from each individual battery cage \((n = 5)\) and treated with 25(OH)D at doses of 6.25, 25, 50, and 100 μg/kg of feed. Samples of the cecal tonsils and liver were collected in RNAlater (Qiagen). After 24 h, RNAlater was discarded, and the organs were stored at −70°C until further analysis. Total RNA was extracted from the different organs and analyzed for relative amounts of IL-1β, IL-10, and 1α-hydroxylase after normalizing for β-actin mRNA levels as described above. The reference group was the group fed 6.25 μg/kg of 25(OH)D and not challenged with coccidial parasite.

Statistical Analysis

For the in vitro study, data were analyzed using a 1-way ANOVA (JMP; SAS Inst. Inc., Cary, NC) to determine the effects of 25(OH)D treatment on dependent variables. For the in vivo study, data were analyzed using a factorial design with factors in the model being 25(OH)D and coccidial parasite infection and their interaction effects. When the main effects were significant \((P < 0.05)\), differences between means were analyzed by Tukey’s least squares means comparison.

RESULTS

Effect of 25(OH)D Treatment on HD11 Cell Nitrite Production and IL-1β mRNA Amounts

At 12 h post-coccidial antigen stimulation, HD11 cells treated with 200 nM of 1,25(OH)\(_2\)D had increased nitrite production \((P < 0.01)\) compared with HD11 cells treated with 0 or 200 nM of 25(OH)D (Fig. 1a). At 12 h post-coccidial antigen stimulation, HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)\(_2\)D had 28- and 14-fold less \((P < 0.01)\) IL-1β mRNA amounts compared with the control group treated with 0 nM of 25(OH)D or 1,25(OH)\(_2\)D (Fig. 1b). At 48 h post-coccidial antigen stimulation, HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)\(_2\)D had no significant differences in IL-1β mRNA amounts compared with the control group (Fig. 1b).

Effect of 25(OH)D Treatment on HD11 Cell, IL-10, and 1α-hydroxylase mRNA Amounts

At 12 h post-coccidial antigen stimulation, treatment of HD11 cells with 25(OH)D decreased IL-10 mRNA by 1.7-fold, but 1,25(OH)\(_2\)D treatment

<table>
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<tr>
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<tr>
<td>DL-methionine</td>
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<td>L-Lysine</td>
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<tr>
<td>Apparent metabolisable energy (Kcal/kg)</td>
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\(^1\)Vitamins and minerals were provided in the form and amount described in the NRC Standard Reference Diet for chickens (NRC, 1994). Experimental diets had 25-hydroxycholecalciferol (HyD; DSM nutritional products, Heerlen, Netherlands) at doses of 6.25, 25, 50, and 100 μg/kg of feed.
increased the amounts of IL-10 mRNA by 2.7-fold ($P < 0.01$) compared with the group treated with 0 nM of 25(OH)D or 1,25(OH)$_2$D (Fig. 2a). At 48 h post-coccidial antigen stimulation, treatment of HD11 cells with 1,25(OH)$_2$D increased the amounts of IL-10 mRNA by approximately 17-fold ($P < 0.01$) compared with the group with 0 nM of 25(OH)D or 1,25(OH)$_2$D (Fig. 2a). At 12 h post-coccidial stimulation, HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)$_2$D had 1.5-fold less ($P < 0.01$) and at 48 h 1.2- to 2.7-fold less ($P < 0.01$) 1α-hydroxylase mRNA amounts compared with the group treated with 0 nM 25(OH)D or 1,25(OH)$_2$D (Fig. 2b).

**Effect of Proliferation on T Cell 1α-hydroxylase and Supernatants from HD11 Cells Treated with 25(OH)D on ConA-B1-VICK IFN-γ mRNA Amounts**

Stimulating primary T cells for 48 h with Con-A decreased ($P = 0.020$) the 1α-hydroxylase mRNA amounts by 3-fold (Fig. 3a). ConA-B1-VICK cells stimulated with 100 nM 1,25(OH)$_2$D or with supernatants from HD11 cells treated with 25(OH)D plus LPS had approximately 1.3-fold less ($P < 0.01$) IFN-γ mRNA compared with the group treated with 25(OH)D (Fig. 3b).

**Effect of 25(OH)D Supplementation on BW Gain and Fecal Oocyst Shedding Post-Coccidia Infection in Layer Birds**

There was a main effect of coccidial oocyst challenge on the BW gain over a 6-d period ($P < 0.01$) in layer birds (Fig. 4a). Compared with the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia pathogen, birds challenged with the coccidia pathogen had approximately 15% reduced BW gain in the groups supplemented with either 6.25, 25, and 50 μg/kg of 25(OH)D but had only 4% reduced BW gain in birds fed 100 μg/kg of 25(OH)D. There was no significant effect of 25(OH)D treatment on the fecal oocyst shedding at d 6 post-coccidial challenge (Fig. 4b).

**Effect of 25(OH)D Supplementation on Cecal Tonsil CD4$^+$, CD8$^+$, and CD4$^+$/CD25$^+$ Percentages Post Coccidial Oocyst Infection in Layer Birds**

Birds fed 100 μg/kg 25(OH)D had decreased ($P = 0.013$) CD8$^+$ cell percentages in cecal tonsils in both coc-
25(OH)D and Coccidiosis in layer chickens

At 15 d post-coccidial oocyst challenge, birds fed 100 μg/kg 25(OH)D and challenged with coccidial oocysts had 17% more CD4+CD25+ cells (P = 0.018) in the cecal tonsil compared with the birds fed 100 μg/kg 25(OH)D and not challenged with coccidial oocysts (Fig. 5b). At 14 d post-coccidial oocyst challenge, CD4+ cell percentages in cecal tonsils of birds fed 25, 50, and 100 μg/kg 25(OH)D and challenged with coccidia oocysts did not differ significantly from that in the control group fed 6.25 μg/kg 25(OH)D.

Effect of 25(OH)D Supplementation on Cecal Tonsil IL-1β, IL-10, and 1α-hydroxylase mRNA Amounts and Liver 1α-hydroxylase mRNA Amounts Post-Coccidia Oocyst Infection in Layer Birds

At 6 d post-coccidial oocyst challenge, among birds challenged with coccidial oocysts, birds fed 25, 50, and 100 μg/kg 25(OH)D had 1.7-, 4.2-, and 3.4-fold numerical decrease in the IL-1β mRNA amounts in the cecal tonsils compared with the birds fed 6.25 μg/kg 25(OH)D (Fig. 6a). The group fed 6.25 μg/kg of 25(OH)D and challenged with coccidial oocysts had no significant difference in the IL-1β mRNA amounts in the cecal tonsils compared with the control birds fed 6.25 μg/kg 25(OH)D and unchallenged with coccidial oocysts (Fig. 6a).

In groups fed 25(OH)D at 6.25, 25, 50, and 100 μg/kg of feed and challenged with coccidial oocysts, IL-10 mRNA amounts increased by approximately 3.3-, 4.5-, 4.9-, and 3.5-fold (P < 0.01) compared with the control group (Fig. 6b). There were no significant differences in the cecal tonsils and liver 1α-hydroxylase mRNA amounts among different treatment groups.

DISCUSSION

Stimulating HD11 cells that were pretreated with either 25(OH)D or 1,25(OH)_{2}D with coccidial antigen decreased 1α-hydroxylase mRNA amounts. We earlier reported that HD11 cells treated in vitro with the bacterial LPS have increased 1α-hydroxylase mRNA (Morris and Selvaraj, 2014). This suggests that coccidial antigens are a poor in vitro inducer of 1α-hydroxylase mRNA in macrophages. In line with prior findings in our lab (Morris and Selvaraj, 2014) and by other researchers (Rockett et al., 1998), 1,25(OH)_{2}D treatment increased nitrite production in HD11 cells, while cells treated with 25(OH)D did not increase nitrite production. Decreased 1α-hydroxylase mRNA amounts post in vitro coccidial antigen stimulation in 25(OH)D-treated cells can explain the inability of 25(OH)D to induce NO production as decreased 1α-hydroxylase will decrease the conversion and subsequent availability of active vitamin D in those cells.

1,25(OH)_{2}D converts naïve T cells into T regulatory cells (Jeffery et al., 2009). T regulatory cells secrete anti-
inflammatory cytokines IL-10 and TGF-β that inhibit the expression of proinflammatory cytokines such as IFN-γ, IL-17, and IL-21 (Gabrysova et al., 2009; Jeffery et al., 2009; Shanmugasundaram and Selvaraj, 2012c). In this study, 1,25(OH)_2D treatment decreased the IFN-γ mRNA content of a T cell line. Prior studies have demonstrated that T cells have limited 1α-hydroxylase activity (Jeffery et al., 2012) and that they rely on neighboring bystander macrophages or dendritic cells for 1,25(OH)_2D. In this study, proliferating T cells decreased the 1α-hydroxylase mRNA amounts, suggesting that proliferating T cells may not be able to efficiently utilize 25(OH)D. Because our previous in vitro studies identified that treating HD11 cells with an inflammatory molecule, LPS, upregulated the 1α-hydroxylase amounts, we hypothesized that treating ConA-B1-VICK cells with supernatants from HD11 cells treated with 25(OH)D and stimulated with LPS suppress the production of proinflammatory cytokines. In this study, T cell lines treated with supernatants from LPS-stimulated macrophage cells and treated with 25(OH)D suppressed the IFN-γ mRNA amounts. T cells treated with supernatants from macrophages that were not stimulated with LPS did not suppress IFN-γ mRNA amounts, showing that chicken T cells, similar to their mammalian counterparts (Jeffery et al., 2012) may not utilize 25(OH)D efficiently as a source of 1,25(OH)_2D.

Both in vitro and in vivo, the 25(OH)D treatment decreased IL-1β mRNA amounts post-coccidial treatment. Parallel to the decreased IL-1β mRNA amounts, there was an increase in the IL-10 mRNA amounts both in vivo and in vitro post-coccidial treatment. Increased cecal tonsil T regulatory cell percentages in birds supplemented with 100 μg/kg of 25(OH)D post-coccidial infection challenge most likely contributed to the increase in the IL-10 in vivo, as Tregs are a natural source of IL-10 (Shanmugasundaram and Selvaraj, 2011, 2012c). In addition to the anti-inflammatory effects of vitamin D mediated through Tregs, vitamin D curtails the inflammatory pathway by upregulating IL-10 in macrophages (Korf et al., 2012). Decreased IL-1β mRNA amounts

Figure 3. Expression of 1α-hydroxylase A) in primary T cells and B) IFN-γ mRNA amounts in ConA-B1-VICK cells treated with 100 nM 1,25-dihydroxycholecalciferol (1,25(OH)2D) or with supernatant from HD11 cells treated with 25-hydroxyvitamin D (25(OH)D) plus lipopolysaccharides (LPS). A) Isolated thymic CD4+ cells (1 × 10^6) were stimulated with 1 μg/mL of Con-A for 48 h. At 48 h post-Con-A stimulation, mRNA amount was analyzed by real-time PCR and normalized to β-actin and compared to the 0-h group so that all bars represent fold change compared to the 0-h group. P values: 1α-hydroxylase: P = 0.020, n = 3. B) ConA-B1-VICK cells (2 × 10^7) were grown in a medium containing either 200 nM of 25(OH)D, 100 nM of 1,25(OH)2D, or with 1:5 dilution of supernatants from HD11 cells treated with 25(OH)D in the presence or absence of an LPS stimulation and stimulated with 7.5 μg/mL of Con-A for 120 h. At 120 h post Con-A stimulation, the mRNA amount was analyzed by real-time PCR and normalized to β-actin and compared to the group treated with 25(OH)D so that all bars represent fold change compared to this group. Means (±SEM) without a common superscript differ significantly within a time point. P values: IFN-γ: P < 0.01. n = 4 except for the treatment group discussed in materials and methods.
observed both in vitro and in vivo might be due to the anti-inflammatory effects of cytokine IL-10. Although supplementing 25(OH)D increased the mRNA amounts of IL10, an anti-inflammatory cytokine, feeding 25(OH)D increased CD8+ cell percentage in the cecal tonsils. This is interesting in the context of a coccidial infec-
tion because early studies have demonstrated a role for CD8+ T cells in acting as transporters for sporozoite and increasing the fecal oocyst shedding (Trout and Lillehoj, 1995). Since our data from fecal oocyst counts are inconclusive to support this argument, more studies need to be done using 25(OH)D to investigate the effects of 25(OH)D on CD8+ T cells and its relative contribution in fecal oocyst shedding. In our earlier study, we observed that supplementing with higher doses of 25(OH)D in broiler diets increased BW gain and decreased expression of the IL-1β mRNA post LPS injection (Morris et al., 2014). In this study, the highest BW gain over a 6-d period and decreased IL-1β mRNA amounts were observed only in the group supplemented with a dose of 25(OH)D above 25 μg/kg. Here we propose high doses of 25(OH)D supplementation in layer birds as a strategy to direct the immune response toward an anti-inflammatory nature. The decreased expression of IL-1β and IFN-γ is due to a synergistic action by cells from both innate and adaptive arms of the immune system involving macrophages and Tregs and the cytokine IL-10. Even though an initial inflammatory response is needed to combat pathogens, an excess inflammatory response in birds comes with a decrease in BW gain (Klasing, 2004).

In conclusion, our current findings suggest a unique nutritional intervention strategy of supplementing with a high dose of 25(OH)D to decrease the production losses associated with a coccidial infection.

**LITERATURE CITED**


