Intramammary 25-hydroxyvitamin D₃ treatment modulates innate immune responses to endotoxin-induced mastitis

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ABSTRACT

Vitamin D signaling in response to pathogen-associated molecules contributes to activation of innate immune responses of bovine monocytes. We hypothesized that lipopolysaccharide (LPS) of bacteria associated with mastitis in dairy cows activates the vitamin D pathway in innate immune cells of the udder and that increasing availability of 25-hydroxyvitamin D₃ [25(OH)D₃] would augment expression of vitamin D-associated genes. The objective of this experiment was to determine the effects of intramammary LPS and 25(OH)D₃ treatments on activation of the vitamin D pathway and innate immune responses of mammary immune cells. Individual mammary quarters of 5 lactating cows were treated with placebo control, 100 µg of 25(OH)D₃, 5 µg of LPS, or a combination of 100 µg of 25(OH)D₃ and 5 µg of LPS. Somatic cells from milk were evaluated for percentage of neutrophil and macrophage populations and expression of genes associated with vitamin D metabolism and innate immunity. Data from samples collected from 4 to 12 h after challenge were analyzed for main effects of LPS and 25(OH)D₃ treatments, treatment interactions, and simple effects of 25(OH)D₃ treatment. Data from samples collected at the time of challenge were used as covariates. The percentages of neutrophils in milk at 8 h postchallenge were 58 ± 10, 82 ± 11, 89 ± 10, and 63 ± 10% of total cells in milk from control, 25(OH)D₃, LPS, and LPS plus 25(OH)D₃ glands, respectively, such that the interaction of LPS and 25(OH)D₃ was significant. Expression of the vitamin D 1α-hydroxylase (*CYP27B1*) and vitamin D receptor genes was upregulated by LPS treatment in total cells, macrophages, and neutrophils in milk. In addition, expression of the vitamin D 24-hydroxylase (*CYP24A1*) gene in milk somatic cells was upregulated

by 25(OH)D₃ and LPS treatments. The inducible nitric oxide synthase (*iNOS*), chemokine (C-C-motif) ligand 5 (*CCL5*), β-defensin 3 (*DEFB3*), *DEFB7*, and *DEFB10* genes were upregulated by LPS treatment in total cells and neutrophils from milk. Expression of *iNOS* in milk somatic cells tended to be affected by the interaction between LPS and 25(OH)D₃, such that 25(OH)D₃ tended to increase *iNOS* in the absence of LPS but not in the presence of LPS. Furthermore, expression of *CCL5* in macrophages was downregulated by 25(OH)D₃. In conclusion, intramammary endotoxin challenge activates the vitamin D pathway in mammary macrophages and neutrophils, and intramammary 25(OH)D₃ treatment alters the percentage of neutrophils and expression of immune genes in milk somatic cells.

Key words: vitamin D, innate immunity, mastitis, dairy cow

INTRODUCTION

Mastitis in dairy cattle is a major concern in regards to animal welfare, milk quality, and profitability for dairy producers, and it eventually affects consumers (Ruegg, 2012). Mastitis is almost always caused by infection of the mammary gland, and environmental bacterial pathogens such as *Escherichia coli* and *Streptococcus uberis* are the most common agents in farms that have adopted control measures for mastitis. The innate response of the mammary gland to a bacterial pathogen triggers an influx of immune cells, primarily neutrophils, and production of inflammatory cytokines and bactericidal molecules (Rainard and Riollot, 2006). Although the innate immune response to bacterial infections of the mammary gland has been well-characterized, a better understanding of the mechanisms influencing innate defenses of the mammary gland is needed to improve treatment and prevention efforts for mastitis.

Several reports using models of cattle, human, and rodent innate immune responses have documented a role for vitamin D in the innate immune response to pathogens. Specifically in cattle, pathogen-associated

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molecules such as LPS and peptidoglycan stimulated expression of the 25-hydroxyvitamin D 1 α -hydroxylase (CYP27B1) in cultures of peripheral blood monocytes (Nelson et al., 2010b, 2011). The CYP27B1 enzyme catalyzes conversion of 25-hydroxyvitamin D₃ [25(OH)D₃] to the active vitamin D hormone, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. The 1,25(OH)₂D₃ primarily acts to regulate gene expression upon its association with intracellular vitamin D receptors (VDR). Synthesis of 1,25(OH)₂D₃ from 25(OH)D₃ in LPS-stimulated cultures of bovine monocytes upregulated expression of β -defensin (*DEFB*) 3, *DEFB*4, *DEFB*6, *DEFB*7, *DEFB*10, inducible nitric oxide synthase (*iNOS*), and chemokine (C-C motif) ligand 5 (*CCL5*) genes (Nelson et al., 2010b; Merriman et al., 2015). Altogether, these data indicate a potential for targeted enhancement of defense against bacterial infections via the vitamin D pathway.

In regards to mastitis, a role for vitamin D signaling in defense of the mammary gland has been indicated in dairy cows. Mammary tissue and somatic cells isolated from milk of quarters experimentally infected with *S. uberis* had increased expression of *CYP27B1*, *VDR*, and the gene for the 25-hydroxyvitamin D 24-hydroxylase (*CYP24A1*) compared with healthy control quarters (Nelson et al., 2010a). Furthermore, Lippolis et al. (2011) tested the effects of intramammary 25(OH)D₃ treatment on the outcomes of experimental *S. uberis* mastitis on the basis that vitamin D signaling is activated during mastitis and observed reduced signs of mastitis in cows treated with intramammary 25(OH)D₃. The authors proposed that intramammary 25(OH)D₃ treatments enhanced defense against *S. uberis* infection by increasing immune cell 1,25(OH)₂D₃ synthesis in the infected quarters. Indeed, the 1,25(OH)₂D₃ metabolite does influence expression of vitamin D-associated genes in the mammary gland (Merriman et al., 2017). However, the influence of 25(OH)D₃ on mammary immune responses to bacterial pathogens has not been determined.

Intramammary LPS challenge induces acute mastitis in dairy cows and has been used as a model to characterize the innate immune response to bacterial pathogens (Bannerman et al., 2003; Schmitz et al., 2004). We hypothesized that intramammary LPS would stimulate CYP27B1 activity in the mammary gland and that simultaneous intramammary 25(OH)D₃ would enhance immune responses associated with vitamin D signaling in cattle. Therefore, the objective of this experiment was to determine the effects of intramammary LPS and 25(OH)D₃ treatments on activation of the vitamin D pathway and innate immune responses of the mammary gland.

MATERIALS AND METHODS

Animals

Five healthy lactating Holstein cows at the University of Florida's Dairy Unit (Hague, FL) were used for this experiment. Cows were between 300 and 680 DIM, not pregnant, free of mastitis (milk SCC $\leq$ 200,000 cells/mL), and otherwise healthy by appearance. Cows were milked twice per day and fed a diet formulated to meet the needs of lactating dairy cows (NRC, 2001). The diet was fed as a TMR and was estimated to provide approximately 40,000 IU of vitamin D₃/d, which is normal for dairy cows in the United States and typically achieves between 50 and 80 ng/mL of 25(OH)D₃ in serum (Nelson et al., 2016a). The University of Florida's Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures for the care and treatment of the animals.

Intramammary LPS and 25(OH)D₃ Treatment

The 25(OH)D₃ was purchased in crystalline form from Cayman Chemical (Ann Arbor, MI) and dissolved to a concentration of 10 mg/mL in ethanol. The concentration and purity of 25(OH)D₃ was confirmed by UV absorption spectroscopy at 228- and 264-nm wavelengths using a molar extinction coefficient of 18,200 M⁻¹cm⁻¹. For intramammary 25(OH)D₃ treatments, 100 μ g of 25(OH)D₃ was diluted in 10 mL of sterile PBS (Dulbecco's PBS without calcium or magnesium; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum as a carrier for 25(OH)D₃ (characterized serum; Hyclone). The LPS from *E. coli* 026:B6 was purchased in crystalline form from Sigma-Aldrich (St. Louis, MO) and dissolved to a concentration of 5 mg/mL in sterile H₂O. Ten-milliliter solutions of PBS containing 5 μ g of LPS (**LIPO**), 100 μ g of 25(OH)D₃ (**25D**), 5 μ g of LPS and 100 μ g of 25(OH)D₃ (**LIPO+25D**), or no LPS or 25(OH)D₃ (control) were prepared for intramammary treatment. The amount of 25(OH)D₃ used was selected on the basis of previous research that showed it did not alter concentrations of 25(OH)D₃ in serum and had inhibitory effects on *S. uberis* mastitis (Lippolis et al., 2011).

Experimental Design

The experiment was a randomized complete block design with a factorial arrangement of treatments. Individual mammary quarters of each cow were considered the experimental units, and cow was the blocking factor. The model used gland-specific responses as previ-

ously shown with LPS models (Schmitz et al., 2004). The control, 25D, LIPO, and LIPO+25D solutions were randomly assigned to each quarter of the 5 cows and aseptically administered via a teat cannula. The quarters administered LPS+25D treatments received repeated treatments with 100 µg of 25(OH)D₃ solution at 12, 24, and 36 h following initial challenge to test the effects of 25-hydroxyvitamin D₃ on the duration of the mammary response to LPS. Accordingly, data collected after 12 h were analyzed separately from data collected from 0 to 12 h.

Body Temperature and Milk SCC Determination

Rectal temperatures were measured at 0, 4, 8, 12, 24, 36, 48, 72, and 96 h to assess the febrile response. The SCC in milk were determined at 0, 12, 24, 36, 48, 72, and 96 h relative to challenge. Prior to full milk withdrawal, foremilk samples were collected into 48-mL collection tubes that contained bronopol as a preservative. Samples were then shipped overnight to Belleview Lab Southeast Milk Inc. (Belleview, FL) for determination of SCC by flow cytometry. Somatic cell counts were log-transformed for statistical analysis and reported as log₁₀ of SCC.

Collection of Milk Somatic Cells and Blood Leukocytes for Gene Expression

Fifty milliliters of milk was collected from each gland at 0, 4, 8, 12, 24, 36, 48, and 72 h relative to the challenge for analysis of gene expression in milk somatic cells. The milk samples collected at 12-h intervals were collected before normal milking. An additional 50 mL of milk was collected at 0 and 8 h for analysis and separation of macrophages and neutrophils. Milk samples were collected into 50-mL conical tubes before full milk withdrawal and transported to the laboratory on ice. Samples were centrifuged at $1,500 \times g$ for 20 min to pellet the cells. The cell pellet was then resuspended in 10 mL of PBS and centrifuged again to wash the somatic cells. The cells were placed in Trizol Reagent (Life Technologies, Carlsbad, CA) and stored at -80°C for later RNA isolation. Blood was sampled from coccygeal vessels into 8-mL lithium heparin evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) at 0 and 8 h relative to the treatment for isolation of monocytes and neutrophils.

For the isolation of macrophages and neutrophils from milk and blood, total somatic cells were isolated from 50-mL milk samples as above, and total blood leukocytes were collected from 1 mL of heparinized blood

by hypotonic lysis of erythrocytes. Cells from milk and blood were then incubated for 30 min on ice with allophycocyanin-conjugated anti-CD14 (Tük4 clone, Life Technologies) and fluorescein isothiocyanate-conjugated mouse anti-bovine CD11b (CC126 clone; AbD Serotec, Raleigh, NC) in 100 µL of PBS containing 0.5% bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) and 2 mM EDTA (Fisher Scientific). Cells were then sorted on the basis of size, granularity, and intensity of fluorescein isothiocyanate and allophycocyanin fluorescence as previously described (Merriman et al., 2017) using a Sony SH800 cell sorter equipped with 405-, 488-, and 633-nm lasers (Sony Biotechnology Inc., San Jose, CA). The CD11b⁺CD14⁺ cells were considered milk macrophages or blood monocytes, and CD11b⁺CD14⁻ cells were considered neutrophils. At least 10,000 CD11b⁺CD14⁺ cells and 50,000 CD11b⁺CD14⁻ cells from each sample were collected with >95% purity. The sorted cells were then pelleted and lysed with RNA lysis buffer from Quick-RNA MiniPrep (Zymo Research, Irvine, CA) for later RNA isolation.

Measurement of Gene Expression

Total milk somatic cell RNA was processed using Trizol Reagent (Life Technologies) according to the manufacturer's instructions and resuspended with 50 µL of RNase-free water. The RNA concentration and quality were assessed by measuring absorbance at 260 and 280 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA). Samples were diluted with RNase-free water to obtain an RNA concentration of 100 ng/µL. The RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit with RNAase inhibitor (Life Technologies) in a 20-µL reaction that contained 10 µL of RNA sample. Reactions were incubated at 23°C for 10 min, followed by 2 h at 37°C and 5 min at 85°C . The cDNA samples were diluted 1:10 with DNase-free water and stored at -20°C . Quantitative PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with gene-specific primers listed in Table 1. Amplifications were performed in a 20-µL reaction that contained 10 µL of SYBR Select qPCR master mix (Life Technologies), 0.5 µL each of 10 µM forward and reverse primers, and 9 µL of the 1:10 diluted cDNA sample. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

For determination of relative transcript abundance, the threshold cycle (C_t) for each gene in each cDNA sample was normalized to the reference gene ribosomal

Table 1. Quantitative PCR primer sequences

Gene ¹	Accession no. ²	Strand ³	Sequence (5'-3')
<i>DEFB3</i>	NM_001282581.1	F	CTCCTCGCACTCCTCTTCCT
		R	GCATCTTCGCCCTTCTTCTACACGA
<i>DEFB4</i>	NM_174775.1	F	TCCTCGCAGTCCTCTTCCT
		R	GGCACAAGAACGGAATACAGA
<i>DEFB5</i>	NM_001130761.1	F	CTCCTCGTGCTCCTCTTCC
		R	ACGGCAGCTTTGAGGATTT
<i>DEFB7</i>	NM_001102362.2	F	TCTTCCTGGTCCTGTCTGCT
		R	GGTGCCAATCTGTCTCCTGT
<i>DEFB10</i>	NM_001115084.1	F	CTCCTCCTGCTCCTCTTGGT
		R	TGCCAATCTGTCTCATGCGT
<i>CCL5</i>	NM_175827	F	CACCCACGTCCAGGAGTATT
		R	CTCGCACCCACTTCTTCTCT
<i>CYP27B1</i>	NM_001192284.1	F	TGGGACCAGATGTTTGCATTCCG
		R	TTCTCAGACTGGTTCCTCATGGCT
<i>CYP24A1</i>	NM_001191417.1	F	GAAGACTGGCAGAGGGTCAG
		R	CAGCCAAGACCTCGTTGATT
<i>IL1B</i>	NM_174093	F	GCCTTGGGTATCAAGGACAA
		R	TTTGGGGTCTACTTCCTCCA
<i>IL8</i>	NM_173925	F	TGCTCTCTGCAGCTCTGTGT
		R	GGTGGAAGGTGTGGAATGT
<i>iNOS</i>	NM_001076799.1	F	GATCCAGTGGTTCGAACCTGC
		R	CAGTGATGGCCGACCTGATG
<i>RPS9</i>	NM_001101152.2	F	GTGAGGTCTGGAGGGTCAAA
		R	GGGCATTACCTTCGAACAGA
<i>VDR</i>	NM_001167932.1	F	GAGGGGAACCGTCCTTTGAG
		R	GAGAAGCTGGTTGGCTCCAT

¹Primer sequences for transcripts of bovine β -defensins (*DEFB3*, *DEFB4*, *DEFB5*, *DEFB7*, *DEFB10*), chemokine (C-C motif) ligand 5 (*CCL5*), 25-hydroxyvitamin D 1 α -hydroxylase (*CYP27B1*), 25-hydroxyvitamin D 24-hydroxylase (*CYP24A1*), IL-1 β (*IL1B*), IL-8 (*IL8*), inducible nitric oxide synthase (*iNOS*), ribosomal protein S9 (*RPS9*), and vitamin D receptor (*VDR*). Sequences have been published previously.

²Accession numbers from National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>).

³F = forward; R = reverse.

protein S9 (*RPS9*) using the formula $C_t(\text{gene of interest}) - C_t(RPS9) = \Delta C_t$ (Merriman et al., 2017). The ΔC_t values for each gene were used for statistical analysis described below. The mean ΔC_t ($\pm$ SE) for each treatment was transformed using the formula $1,000 \times 2^{-\Delta C_t}$ to report the relative number of transcripts per 1,000 *RPS9* transcripts.

Measurement of 25(OH)D Concentrations

Combined concentrations of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 [**25(OH)D**] in serum and milk samples were measured using a commercially available ELISA (Human VD₃ ELISA, Eagle Biosciences, Nashua, NH) using a 25(OH)D standard curve (5 ng/mL to 125 ng/mL) prepared in bovine serum as previously described (Nelson et al., 2016b). Measurement of absorbance at 450 nm was made using a BioTek Synergy plate reader (BioTek Instruments Inc., Winooski, VT). Inter- and intra-assay coefficients of variation for the ELISA were 5.6 and 4.4%, respectively.

Statistical Analysis

All data were analyzed using the ΔC_t values for each gene in SAS version 9.4 (SAS Institute Inc., Cary, NC). The experiment was analyzed as a repeated-measures analysis with the MIXED procedure of SAS. The model accounted for fixed effects of treatment, time, and interaction between treatment and time and the random effects of gland nested within cow and treatment. The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F-tests in the statistical models. Measurements collected at 0 h for each variable were used as covariates in the analysis. Data from 4 to 12 h were used to determine main effects of LPS or 25(OH)D₃ treatments and the interactions between LPS and 25(OH)D₃. Data collected after 12 h were analyzed only for simple effects of 25D or LPS treatments, as glands treated with LIPO+25D received repeated injections of 25(OH)D₃ at 12, 24, and 36 h. Tests for multiple comparisons were made with the Tukey-Kramer adjustment. Statistical significance was declared at $P \leq 0.05$.

and a tendency for significance was declared at $0.05 < P \leq 0.10$.

RESULTS

Clinical Responses to Intramammary LPS and 25(OH)D₃ Treatments

Concentrations of 25(OH)D in serum were 43 ± 7 ng/mL (range: 27–51 ng/mL for all cows) and did not change over time (Figure 1A). Concentrations of 25(OH)D were measured in samples of 2 of the 5 cows and were below the limit of detection (5 ng/mL) in quarters not treated with 25(OH)D but near that of

serum in quarters treated with 25D and LPS+25D at 4 h (Figure 1A). Rectal temperatures increased to 40.5°C on average within 4 h after the LPS challenge and were below 39°C by 12 h after challenge (Figure 1B).

The SCC in milk of LPS and LIPO+25D quarters was elevated (7.24×10^6 and 6.76×10^6 cells/mL) compared with control and 25D quarters (1.20×10^5 and 2.24×10^5 cells/mL) at 12 h after treatment (main effect of LPS, $P < 0.001$; Table 2; Figure 2A). The SCC of the 25D quarters tended ($P = 0.09$) to increase compared with control glands at 12 h (Table 2), but it did not differ between LIPO and LIPO+25D quarters. Overall, from 12 to 72 h postchallenge, the SCC in milk from 25D, LIPO, and LIPO+25D quarters was

Figure 1. Least squares means $\pm$ SEM of (A) combined concentrations of 25(OH)D₂ and 25(OH)D₃ [25(OH)D] in serum or milk of treatment quarters, (B) rectal temperatures, (C) log₁₀ of milk SCC, and (D) percentages of macrophages and neutrophils in milk of cows ($n = 5$) treated intramammary with the control (CNTRL), 100 μ g of 25-hydroxyvitamin D₃ (25D), 5 μ g of LPS (LIPO), or 5 μ g of LPS in combination with 100 μ g of 25-hydroxyvitamin D₃ (LIPO+25D). (A) Concentrations of 25(OH)D in milk represent data from 2 of 5 cows. Concentrations of 25(OH)D in milk of CNTRL and LIPO quarters were below limit of detection (5 ng/mL). (C) Means of LIPO and LIPO+25D quarters differed from those of CNTRL quarters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$) at indicated time points. (D) Proportions of CD11b⁺CD14⁺ (macrophages) and CD11b⁺CD14⁻ (neutrophils) cells in milk measured at 8 h postchallenge. Summary statistics for data in panels C and D are provided in Table 2.

elevated ($P < 0.01$) compared with milk from control quarters (Figure 1B) but did not differ between LIPO and LIPO+25D quarters (Figure 1C).

The percentages of macrophages in milk at 8 h postchallenge were decreased in LIPO and LIPO+25D quarters (4.6 and 2.9%, respectively) compared with control and 25D quarters (14.2 and 8.7%, respectively; LPS main effect, $P = 0.005$; Table 2; Figure 1D), but percentages did not differ between control and 25D quarters or LIPO and LIPO+25D quarters. The percentages of neutrophils in milk at 8 h postchallenge (58.4, 82.1, 88.7, and 62.8% in control, 25D, LIPO, and LIPO+25D quarters, respectively) were altered by treatments such that main effects of LPS and 25(OH)D₃ treatments were not significant, but the interaction between LPS and 25(OH)D₃ treatments was significant ($P = 0.027$; Table 2; Figure 1D).

Effects of Intramammary LPS and 25(OH)D₃ on Vitamin D Pathway Genes

The LPS challenge induced a rapid upregulation of the vitamin D pathway genes in total somatic cells isolated from milk of LIPO and LIPO+25D quarters compared with the control. Transcripts for *CYP27B1* and *VDR* genes were upregulated in the LIPO and LIPO+25D quarters compared with control and 25D quarters beginning at 4 h and remained elevated until 12 h ($P < 0.001$; Table 3; Figure 2A). Maximum *CYP27B1* expression was almost 90-fold greater in LIPO and LIPO+25D quarters at 8 h compared with baseline expression at 0 h (Figure 2A). Maximum *VDR* expression was 6-fold higher in LIPO and LIPO+25D quarters at 4 h compared with baseline at 0 h (Figure 2A). Expression of *CYP24A1* increased ($P < 0.05$) in milk somatic cells from 25D, LIPO, and LIPO+25D quarters compared with control quarters (Figure 2A). Overall, LPS and 25(OH)D₃ treatments increased (P

< 0.05) expression of *CYP24A1* in milk somatic cells (Table 3). A tendency ($P = 0.06$) for interaction between LPS and 25(OH)D₃ treatments also was observed for *CYP24A1* expression (Table 3).

For macrophage and neutrophil populations within milk somatic cells, LPS treatment upregulated ($P < 0.05$) expression of *CYP27B1* and *VDR* in macrophages and neutrophils (Table 3; Figure 2B). Expression of *CYP24A1* in macrophages and neutrophils from milk, however, was not altered by LPS and 25(OH)D₃ treatments (Table 3; Figure 2B).

Compared with peripheral blood monocytes, *CYP27B1*, *VDR*, and *CYP24A1* were greater ($P < 0.01$) in milk macrophages from LIPO glands at 8 h postchallenge (Figure 3A). Expression of *CYP27B1* and *VDR* also were greater ($P < 0.01$) in macrophages in milk compared with blood monocytes before LPS challenge (Figure 3A). Similarly, *CYP27B1* and *VDR* transcripts were greater ($P < 0.05$) in milk neutrophils from LIPO quarters compared with blood neutrophils at 8 h postchallenge, but *CYP24A1* in milk neutrophils did not differ from blood neutrophils (Figure 3B).

Effects of Intramammary LPS and 25(OH)D₃ on Expression of Immune Genes

All immune genes analyzed in milk somatic cells, except for *DEFB5*, were upregulated ($P < 0.05$) by LPS treatment (Table 3; Figure 4). Treatment with 25(OH)D₃ did not influence expression of any of the immune genes in milk somatic cells (Table 3; Figure 4) except for *iNOS*, which tended to increase ($P = 0.1$) in 25D quarters compared with the control (Figure 4). Also, a tendency for the effect of interaction ($P = 0.08$) between LPS and 25(OH)D₃ treatments was observed for expression of *iNOS* (Table 3). Expression of *iNOS*, *DEFB3*, *DEFB4*, *DEFB7*, and *DEFB10* peaked from 8 to 12 h postchallenge, whereas that of *IL1B*

Table 2. Effect (means) of intramammary LPS and 25-hydroxyvitamin D₃ on milk SCC and proportions of macrophages and neutrophils

Variable	Treatment ¹				SEM	P-value ²		
	Control	25D	LIPO	LIPO+25D		25(OH)D ₃	LPS	LPS × 25(OH)D ₃
SCC, ³ log ₁₀ cells/mL	5.08 ^a	5.35 ^a	6.86 ^b	6.83 ^b	0.10	0.178	<0.001	0.275
Macrophages, ⁴ %	14.2 ^a	8.7 ^{ab}	4.6 ^b	2.9 ^b	2.4	0.145	0.005	0.418
Neutrophils, ⁴ %	58.4 ^a	82.1 ^{ab}	88.7 ^b	62.8 ^{ab}	9.9	0.907	0.590	0.028

^{a,b}Means within a row with different letters are different ($P < 0.05$).

¹Treatments: Control = 10 mL of PBS with 10% fetal bovine serum; 25D = 100 µg of 25-hydroxyvitamin D₃; LIPO = 5 µg of LPS; LIPO+25D = 5 µg of LPS plus 100 µg of 25-hydroxyvitamin D₃.

²25(OH)D₃ = effect of 25-hydroxyvitamin D₃ (control and LIPO vs. 25D and LIPO+25D); LPS = effect of LPS treatment (control and 25D vs. LIPO and LIPO+25D); LPS × 25(OH)D₃ = interaction between LPS and 25-hydroxyvitamin D₃ treatments (control and LIPO+25D vs. 25D and LIPO).

³Somatic cell counts in milk at 12 h postchallenge.

⁴Percentages of macrophages and neutrophils in milk measured at 8 h postchallenge.

and *IL8* peaked at 4 to 8 h postchallenge in LIPO and LIPO+25D quarters (Figure 4). In contrast, expression of *DEFB5* decreased ($P < 0.05$) at 4 h postchallenge in milk somatic cells from LIPO and LIPO+25D quarters (Figure 4).

Treatment with LPS increased ($P < 0.001$) expression of *iNOS* and tended to increase expression of *DEFB3*, *DEFB10*, and *IL1B* in macrophages in milk somatic cells (Table 3). Expression of *CCL5* in macrophages, on the other hand, was decreased ($P < 0.05$) by 25(OH)D₃ treatment (Table 3; Figure 5). Treatment with LPS also upregulated ($P < 0.05$) *iNOS*, *CCL5*, *DEFB3*, *DEFB4*, *DEFB7*, *DEFB10*, and *IL1B* in neutrophils from milk

(Table 3; Figure 5). Except for *CCL5* in macrophages, the 25(OH)D₃ treatment did not alter expression of the immune genes analyzed in macrophages or neutrophils, and no interactions were observed between LPS and 25(OH)D₃ treatments (Table 3).

Effects of Repeated Intramammary 25(OH)D₃ in LPS-Challenged Quarters

Expression of *CYP27B1* in total milk somatic cells remained elevated ($P < 0.05$) in LIPO and LIPO+25D quarters compared with control quarters until 24 h postchallenge, whereas *VDR* expression was not greater

Figure 2. Abundance (LSM $\pm$ SEM) of transcripts for (A) vitamin D pathway genes *CYP27B1*, *VDR*, and *CYP24A1* in total somatic cells and (B) macrophages and neutrophils from milk of glands treated with the control (CNTRL), 100 μ g of 25-hydroxyvitamin D₃ (25D), 5 μ g of LPS (LIPO), or 5 μ g of LPS in combination with 100 μ g of 25-hydroxyvitamin D₃ (LIPO+25D); $n = 5$ /treatment. Transcript abundances are expressed relative to *RPS9* transcript abundance. Macrophages and neutrophils were isolated at 8 h postchallenge. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; relative transcript abundances in LIPO and LIPO+25D quarters differed from CNTRL. P -values for main effects of treatments and treatment interactions are provided in Table 3.

in LIPO and LIPO+25D quarters compared with control quarters from 24 to 72 h (Figure 6). Expression of *CYP24A1* in milk somatic cells did not differ between LIPO and control quarters from 12 to 72 h postchallenge, but it was greater ($P < 0.01$) in somatic cells of LIPO+25D quarters than in somatic cells of control or LIPO quarters (Figure 6). Expression of *iNOS* in somatic cells from LIPO and LIPO+25D quarters remained elevated ($P < 0.05$) at 24 and 36 h compared with the control but did not differ between LIPO and LIPO+25D quarters (Figure 6). The β -defensins, *CCL5*, *IL1B*, and *IL8* genes were not elevated in LIPO or LIPO+25D quarters compared with control quarters from 24 to 72 h postchallenge, and none of the genes differed in 25D quarters compared with control quarters from 24 to 72 h (data not shown).

DISCUSSION

Understanding mechanisms that control immune responses in the mammary gland is necessary to improve treatment and prevention strategies for mastitis in dairy cows. Previous reports have demonstrated that vitamin D pathway signaling (*CYP27B1*, *VDR*, and *CYP24A1*) is upregulated in the mammary gland during *S. uberis* mastitis (Nelson et al., 2010a) and that intramammary 25(OH) D_3 treatment inhibited *S. uberis* mastitis in dairy cattle (Lippolis et al., 2011). Here, we observed that vitamin D pathway signaling was rap-

idly induced by the innate response to intramammary LPS challenge. Although intramammary 25(OH) D_3 modulated milk SCC and proportion of neutrophils in milk, 25(OH) D_3 did not influence expression of immune genes in the same way as in LPS-stimulated monocyte cultures (Nelson et al., 2010b; Merriman et al., 2015). This information aids in understanding the dynamics of vitamin D signaling during the innate immune response to bacterial endotoxins.

The LPS challenge induced a large increase in expression of *CYP27B1*, the gene that encodes for the 25-hydroxyvitamin D 1 α -hydroxylase, in milk macrophages and neutrophils. Induction of vitamin D pathway signaling in macrophages via toll-like receptor recognition of bacterial associated molecules such as LPS has been demonstrated using in vitro cultures of monocytes and macrophages (Liu et al., 2006; Nelson et al., 2010b). Recognition of LPS via the CD14/TLR4 receptor complex activates mitogen-activated protein kinase and nuclear factor κ B signaling pathways, which have been implicated in the induction of *CYP27B1* transcription (Stoffels et al., 2006). The relative abundance of *CYP27B1* transcripts was similar between macrophages and neutrophils upon LPS stimulation, unlike the predominant expression of *CYP27B1* in macrophages previously reported for *S. uberis* mastitis (Nelson et al., 2010a). Neutrophils make up almost 90% of total somatic cells during mastitis and therefore potentially are a major contributor to 1,25(OH) $_2D_3$ synthesis in

Table 3. Effect of intramammary LPS and 25-hydroxyvitamin D_3 on gene expression in milk somatic cells, macrophages, and neutrophils¹

Gene ²	Total cells ³			Macrophages ⁴			Neutrophils ⁴		
	LPS	25(OH) D_3	LPS $\times$ 25(OH) D_3	LPS	25(OH) D_3	LPS $\times$ 25(OH) D_3	LPS	25(OH) D_3	LPS $\times$ 25(OH) D_3
<i>CYP27B1</i>	<0.001	0.400	0.853	0.003	0.759	0.917	0.002	0.864	0.727
<i>VDR</i>	0.003	0.956	0.721	0.038	0.526	0.316	0.004	0.761	0.163
<i>CYP24A1</i>	0.048	0.049	0.057	0.622	0.760	0.181	0.848	0.737	0.573
<i>iNOS</i>	<0.001	0.562	0.081	0.006	0.307	0.904	<0.001	0.287	0.603
<i>CCL5</i>	0.001	0.940	0.640	0.702	0.028	0.522	0.002	0.772	0.767
<i>DEFB3</i>	0.021	0.678	0.351	0.061	0.481	0.272	<0.001	0.346	0.646
<i>DEFB4</i>	0.269	0.667	0.827	0.119	0.850	0.489	0.044	0.527	0.560
<i>DEFB5</i>	0.930	0.827	0.763	0.895	0.765	0.401	0.156	0.856	0.982
<i>DEFB7</i>	0.005	0.924	0.747	0.101	0.628	0.744	0.001	0.337	0.452
<i>DEFB10</i>	0.004	0.610	0.748	0.077	0.924	0.595	<0.001	0.963	0.473
<i>IL1B</i>	<0.001	0.907	0.749	0.066	0.827	0.417	<0.001	0.246	0.959
<i>IL8</i>	<0.001	0.859	0.728	0.272	0.728	0.098	0.149	0.886	0.947

¹Values represent P -values for effects of treatments on abundances of listed transcripts. Treatments: Control = 10 mL of PBS with 10% fetal bovine serum; 25D = 100 μ g of 25-hydroxyvitamin D_3 ; LIPO = 5 μ g of LPS; LIPO+25D = 5 μ g of LPS plus 100 μ g of 25-hydroxyvitamin D_3 . 25(OH) D_3 = effect of 25-hydroxyvitamin D_3 (control and LIPO vs. 25D and LIPO+25D); LPS = effect of LPS treatment (control and 25D vs. LIPO and LIPO+25D); LPS $\times$ 25(OH) D_3 = interaction between LPS and 25-hydroxyvitamin D_3 treatments (control and LIPO+25D vs. 25D and LIPO).

²Transcripts for 25-hydroxyvitamin D 1 α -hydroxylase (*CYP27B1*), 25-hydroxyvitamin D 24-hydroxylase (*CYP24A1*), vitamin D receptor (*VDR*), inducible nitric oxide synthase (*iNOS*), chemokine (C-C motif) ligand 5 (*CCL5*), bovine β -defensins (*DEFB3*, *DEFB4*, *DEFB5*, *DEFB7*, *DEFB10*), IL-1 β (*IL1B*), and IL-8 (*IL8*) were measured by quantitative real-time PCR.

³Comparison of gene expression in total somatic cells isolated from milk sampled at 4, 8, and 12 h postchallenge.

⁴Comparison of gene expression in macrophages and neutrophils isolated from milk sampled at 8 h postchallenge.

Figure 3. Abundance (LSM $\pm$ SEM) of transcripts for vitamin D pathway genes *CYP27B1*, *VDR*, and *CYP24A1* in (A) monocytes or milk macrophages and (B) neutrophils from peripheral blood or milk of quarters that received intramammary LPS ($n = 5/\text{treatment}$). Samples were collected at 0 h (prechallenge) or 8 h (postchallenge) relative to LPS challenge. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; relative transcript abundances in milk cells differed from blood cells.

the mammary gland during the early innate immune response to bacteria.

The simultaneous induction of *VDR* by LPS stimulation is presumed to amplify the effects of 1,25(OH)₂D₃ synthesis. Many cells of the body express the *VDR* (Norman, 2008), but the factors contributing to *VDR* expression are not completely understood. Upregulation of *VDR* expression in response to LPS highlights the potential importance of 1,25(OH)₂D₃ on immune regulation and, together with *CYP27B1*, provides further support that induction of the vitamin D pathway is an integral component of the innate immune response. Notably, *CYP27B1* and *VDR* transcripts also were greater in milk macrophages compared with blood monocytes before the challenge and in glands not

treated with LPS. The baseline level of *CYP27B1* expression in resident macrophages of the gland suggests that some 1,25(OH)₂D₃ synthesis may occur as part of the homeostatic function of tissue macrophages.

The increase of *CYP24A1* expression in response to LPS and 25(OH)D₃ treatments in the present experiment indicates probable function of *CYP27B1* and *VDR* in the mammary gland. The *CYP24A1* encodes for the 25-hydroxyvitamin D 24-hydroxylase, which provides feedback control of vitamin D signaling by metabolizing 25(OH)D₃ and 1,25(OH)₂D₃ to inactive metabolites (Reinhardt et al., 1999). The *CYP24A1* promoter contains several *VDR* binding sites (Meyer et al., 2010), and it was strongly induced by 1,25(OH)₂D₃ in monocytes, neutrophils, and mammary

epithelial cells (Merriman et al., 2015). Intramammary 1,25(OH)₂D₃ treatment also induced a massive increase of *CYP24A1* expression in milk somatic cells (Merriman et al., 2017). In the present experiment, expression of *CYP24A1* was increased by LPS and 25(OH)D₃ treatments in total somatic cells within the first 4 h. We

Figure 4. Abundance (LSM ± SEM) of transcripts for inducible nitric oxide synthase (*iNOS*), chemokine (C-C motif) ligand 5 (*CCL5*), β-defensin (*DEFB*) 3, *DEFB4*, *DEFB5*, *DEFB7*, *DEFB10*, IL-1β (*IL1B*), and IL-8 (*IL8*) in total somatic cells from milk of quarters treated with the control (CNTRL), 100 μg of 25-hydroxyvitamin D₃ (25D), 5 μg of lipopolysaccharide (LIPO), and 5 μg of 25-hydroxyvitamin D₃ in combination with 100 μg of 25-hydroxyvitamin D₃ (LIPO+25D); n = 5/treatment. Transcript abundances are expressed relative to *RPS9* transcript abundance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; relative transcript abundances in LPS (LIPO and LIPO+25D) quarters differed from non-LPS (CNTRL and 25D) quarters. *P*-values for main effects of treatments and treatment interactions are provided in Table 3.

speculate that the increase in *CYP24A1* resulted from increased conversion of 25(OH)D₃ to 1,25(OH)₂D₃ by CYP27B1 in macrophages in neutrophils. Conclusive

evidence of 1,25(OH)D₃ synthesis, however, is limited because accurate measurement of 1,25(OH)₂D₃ in milk is not feasible.

Figure 5. Abundance (LSM ± SEM) of transcripts for inducible nitric oxide synthase (*iNOS*), chemokine (C-C motif) ligand 5 (*CCL5*), β-defensin (*DEFB*) 3, *DEFB4*, *DEFB5*, *DEFB7*, *DEFB10*, IL-1β (*IL1B*), and IL-8 (*IL8*) in CD11b⁺CD14⁺ (macrophages) and CD11b⁺CD14[−] (neutrophils) cells from milk of quarters treated with the control (CNTRL), 100 μg of 25-hydroxyvitamin D₃ (25D), 5 μg of lipopolysaccharide (LIPO), and 5 μg of LPS in combination with 100 μg of 25-hydroxyvitamin D₃ (LIPO+25D); n = 5/treatment. Transcript abundances are expressed relative to *RPS9* transcript abundance at 8 h postchallenge. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; relative transcript abundances differed from CNTRL. *P*-values for main effects of treatments and treatment interactions are provided in Table 3.

We anticipated that intramammary 25(OH)D₃ treatment in combination with LPS would increase *CYP24A1* compared with LPS alone because of increased substrate for CYP27B1. Although treatment with 25(OH)D₃ increased *CYP24A1* in milk somatic cells overall, expression of *CYP24A1* was not greater in LIPO+25D glands compared with LIPO glands within the first 12 h after challenge. In contrast, *CYP24A1* increased in 25D glands compared with control glands. Also, *CYP24A1* was greater in LIPO+25D glands compared with LIPO glands from 12 to 36 h when the acute response was subsiding. The lack of *CYP24A1* response to 25(OH)D₃ in combination with LPS treatment may be attributed to inhibitory effects of LPS stimulation on induction of *CYP24A1* expression by 1,25(OH)₂D₃ (Nelson et al., 2010b). The antagonistic effect of LPS on *CYP24A1* expression is further supported by a tendency for interaction between 25(OH)D₃ and LPS treatments on *CYP24A1* expression in milk somatic cells. Interaction between the VDR and other intracel-

lular pathways activated by TLR signaling has been reported (Vidal et al., 2002; Sadeghi et al., 2006) and potentially explains the antagonistic effect of LPS on *CYP24A1* expression. Taken together, we propose that the induction of *CYP24A1* expression in milk somatic cells by LPS treatment occurred because of increased CYP27B1 and VDR activity; however, no additional increase in *CYP24A1* expression was achieved by the combination of 25(OH)D₃ and LPS treatments because of the antagonistic effect of LPS on *CYP24A1* expression.

Intramammary 25(OH)D₃ treatment increased milk SCC in non-LPS glands and altered the proportions of neutrophils in milk. In comparison, milk SCC did not increase with intramammary 1,25(OH)₂D₃ treatment in healthy glands but tended to increase with 1,25(OH)₂D₃ in subclinically infected glands (Merriman et al., 2017). Although an interaction between LPS and 25(OH)D₃ treatments was observed for proportions of neutrophils in milk at 8 h postchallenge in this experi-

Figure 6. Abundance (LSM $\pm$ SEM) of transcripts for vitamin D pathway genes (*CYP27B1*, *VDR*, and *CYP24A1*) and inducible nitric oxide synthase (*iNOS*) in total somatic cells from milk of quarters at 12 to 72 h after treatment with the control (CNTRL), 5 µg of LPS (LIPO), and 5 µg of LPS in combination with 100 µg of 25-hydroxyvitamin D₃ (LIPO+25D) intramammary treatments; n = 5/treatment. The LIPO+25D quarters received repeated injections of 100 µg of 25-hydroxyvitamin D₃ at 12, 24, and 36 h postchallenge and were compared with CNTRL and LIPO quarters. Transcript abundance is expressed relative to *RPS9*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; relative transcript abundance in LIPO and LIPO+25D quarters differed from CNTRL. #*P* < 0.05, ##*P* < 0.01; relative transcript abundance in LIPO+25D glands differed from LIPO quarters.

ment, the effect of treatments on neutrophil counts in milk is unknown because cell counts were not measured at 8 h postchallenge. Additionally, cells were considered neutrophils if they were CD11b⁺CD14⁻, so the treatment interaction observed could instead be interpreted as a change in cell phenotype rather than a change in relative abundance of neutrophils. In either case, the effects of intramammary 25(OH)D₃ treatment on milk SCC warrant further investigation on the role of vitamin D signaling in immune cell trafficking to the mammary gland.

We anticipated that intramammary 25(OH)D₃ treatment would boost expression of *iNOS*, *CCL5*, and β -defensin genes in milk somatic cells because each of these genes increased with 25(OH)D₃ treatment in LPS-stimulated cultures of peripheral blood monocytes (Nelson et al., 2010b; Merriman et al., 2015). Furthermore, intramammary 1,25(OH)₂D₃ treatment increased expression of *iNOS*, *DEFB4*, and *DEFB7* in milk somatic cells (Merriman et al., 2017) and macrophages, and on the basis of *CYP27B1* expression in the present study, neutrophils likely had the ability to convert 25(OH)D₃ to 1,25(OH)₂D₃. Conversely, *iNOS* and β -defensin expression did not increase in LIPO+25D glands compared with LIPO glands, possibly because the strong acute response to LPS overwhelmed any influence of the vitamin D signal. In addition, contrary to the effects of 25(OH)D₃ in LPS-stimulated monocytes (Nelson et al., 2010b), expression of *CCL5* decreased with 25(OH)D₃ in macrophages. These observations underscore the context and cell-specific responses elicited by vitamin D signaling but do not rule out the influence of vitamin D signaling on expression of *iNOS* and β -defensin genes.

The challenge model used in the present experiment differed from that used by Lippolis et al. (2011), who showed that intramammary 25(OH)D₃ treatment reduced the severity of *S. uberis* mastitis. Inflammatory responses did not develop until 48 to 72 h postchallenge in the challenge model used by Lippolis et al. (2011) compared with 4 to 8 h in the present LPS challenge model. Furthermore, the 100 μ g of 25(OH)D₃ treatments in the experiment reported by Lippolis et al. (2011) were administered daily starting concurrent with the bacterial challenge, before initiation of inflammation of the mammary glands. As observed here, resident macrophages of the healthy mammary gland have increased *CYP27B1* expression compared with blood monocytes, which indicates the potential ability to convert 25(OH)D₃ to 1,25(OH)₂D₃. Indeed, the intramammary 25(OH)D₃ treatment increased *iNOS* in milk somatic cells independent of LPS in this experiment. Thus, the influence of 25(OH)D₃ in noninflammatory conditions may be quite different than when acute in-

flammatory conditions are induced by LPS or other inflammatory mediators.

One consideration of this experiment is that the cows received approximately 40,000 IU of dietary vitamin D₃. Dairy cows fed similar amounts of vitamin D₃ in similar conditions typically have serum 25(OH)D₃ concentrations between 40 and 100 ng/mL (Nelson et al., 2016a). Serum 25(OH)D concentrations of cows in this experiment were 43 ng/mL on average, which is arguably sufficient for support of vitamin D-dependent functions of the immune system. Concentrations of 25(OH)D₃ in milk are typically below 0.5 ng/mL and poorly correlated with 25(OH)D concentrations in serum (McDermott et al., 1985). In this experiment, milk 25(OH)D concentrations were below the limit of detection (5 ng/mL) in samples from quarters not treated with 25(OH)D₃. Intramammary treatment with 100 μ g of 25(OH)D₃ increased milk 25(OH)D₃ concentrations to values near that of serum; however, the amount or route of 25(OH)D₃ injected may not have adequately increased intracellular 25(OH)D₃ concentrations to elicit an observable change in expression of immune genes that were measured.

Another important consideration of the experimental design implemented is that mammary gland within cow was the experimental unit. Systemic changes were observed in cows over the course of the experiment. It is possible that LPS treatments might have influenced responses in glands not treated with LPS. Nevertheless, by using gland within cow as the experimental unit, we minimized the variability among experimental units at the same time that we controlled for the random effect of cow. By default, it is assumed that glands from the same cow are more alike than glands from different cows. Therefore, the current approach used likely improved the precision of the experiment, although it is possible that systemic effects of a treatment might have influenced response to another treatment within the same cow. Other considerations of the experiment were the number of cows used and the timing and methods of sample collections. The number of cows used was the minimum needed to detect changes in gene expression on the basis of power analysis using data from previous experiments, so effects of treatments may have been undetected because the minimum number of cows was used. In regards to sample collections, collection of sequential aliquots of milk from each quarter at each time point for total somatic cell and sorted cell gene expression potentially gives rise to heterogeneous populations of cells for the subsequent assays performed because concentrations of leukocytes in milk are not homogeneous among fractions of milk collected from the same quarter. The cooling of milk samples and simple centrifugation procedure we used for isolation of cells

was to facilitate rapid collection of cells and limit cell degradation, but it comes at the expense of extraction efficiency and failure to collect a full representation of immune cells secreted in the milk. Indeed, much of the activity of the vitamin D pathway may be taking place in neutrophils and macrophages present in the alveolar lumen or extravagating the mammary epithelium.

A secondary goal of this experiment was to determine the dynamics of β -defensin gene expression in response to LPS challenge. Expression of *DEFB3*, *DEFB4*, *DEFB5*, and *DEFB10* has been documented in infected (Kościuczuk et al., 2014) and healthy (Tetens et al., 2010) bovine mammary glands. Lipopolysaccharide treatment induced expression of several β -defensins in mammary epithelial cell and neutrophil cultures (Merriman et al., 2015), but the dynamics and factors controlling their expression in the mammary gland were not known. We observed that LPS stimulated *DEFB3*, *DEFB4*, *DEFB7*, and *DEFB10* in somatic cells within several hours after challenge. In agreement with in vitro experiments, the response of the β -defensins to intramammary LPS also was more dramatic in neutrophils compared with macrophages (Merriman et al., 2015). Although not shown, similar results were observed for expression of the gene for lingual antimicrobial peptide, another member of the bovine chromosome 27 β -defensin gene family, which also was induced by intramammary LPS challenge in a previous experiment (Isobe et al., 2009). Expression of *DEFB5*, on the other hand, decreased at 4 h after LPS challenge, which is similar to the response of monocytes but contrary to the response of mammary epithelial cells and neutrophil cultures (Merriman et al., 2015). Of the β -defensin genes, the response of *DEFB10* was most dramatic, increasing more than 200-fold compared with baseline expression. The *DEFB10* peptide also was the most abundant β -defensin peptide in milk fat globules from glands infected with *Staphylococcus aureus* (Reinhardt et al., 2013), highlighting the potential significance in defense against bacterial infection of the mammary gland.

CONCLUSIONS

Immune cells of the mammary gland are capable of converting 25(OH) D_3 to 1,25(OH) $_2D_3$ as indicated by elevated *CYP27B1* transcript abundance and changes in 1,25(OH) $_2D_3$ -inducible genes in response to intramammary 25(OH) D_3 treatment. The innate immune response to intramammary endotoxin challenge increases vitamin D signaling in mammary immune cells and, although effects of intramammary 25(OH) D_3 treatment were modest, the outcomes of this experiment indicate that availability of 25(OH) D_3 for synthesis of

1,25(OH) $_2D_3$ in mammary immune cells alters mammary immune responses. Intramammary 25(OH) D_3 treatment may not dramatically influence the acute response to endotoxin-induced mastitis; however, harnessing the immunomodulatory effects of vitamin D signaling in immune cells via dietary or therapeutic manipulation of 25(OH) D_3 availability may provide an opportunity to improve protection of the mammary gland from bacterial infections.

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