Neutrophil β -defensin gene expression of postpartum dairy cows is altered by prepartum dietary cation-anion difference

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ABSTRACT

The objective of this study was to evaluate expression of a cluster of genes encoding β -defensin antimicrobial peptides in neutrophils of postpartum cows in relation to prepartum dietary cation-anion difference (DCAD), vitamin D, and postpartum disease. Pregnant dry Holstein cows (28 nulliparous and 51 parous) at 255 d gestation were blocked by parity and randomly assigned to 4 prepartum diets of positive (+130 mEq/kg) or negative (−130 mEq/kg) DCAD and either 3 mg vitamin D₃ or 3 mg of 25-hydroxyvitamin D₃ per 11 kg of dry matter/d. Treatment diets were fed from 255 d of gestation until calving. Peripheral blood neutrophils of 35 parous cows were collected at 0 and 3 d after calving and stimulated with 0 or 100 ng/mL of lipopolysaccharide (LPS). Furthermore, serum Ca and incidences of postpartum diseases were recorded for all cows. The mRNA transcripts of β -defensin genes were quantified by real-time PCR, and data were analyzed with a general linear mixed model to test for fixed effects and interactions of day, level of DCAD, source of vitamin D, and incidence of disease. Effects of DCAD and vitamin D on neutrophil oxidative burst and phagocytosis were previously reported but were analyzed for effects of disease in the present study. Transcripts for *DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB7*, *DEFB10*, and lingual antimicrobial peptide (*LAP*) in neutrophils were upregulated by LPS at 0 d but not at 3 d. Transcripts for *DEFB4* and *DEFB7* in LPS-stimulated neutrophils were greater in cows fed negative DCAD diets compared with positive DCAD. Source of vitamin

D (vitamin D₃ vs. 25-hydroxyvitamin D₃) did not affect expression of β -defensins in neutrophils. Cows with postpartum subclinical hypocalcemia (serum Ca <2.0 mM) had decreased *DEFB3*, *DEFB4*, *DEFB6*, *DEFB7*, *DEFB10*, and *LAP* expression in LPS-stimulated neutrophils compared with cows that did not experience subclinical hypocalcemia. Likewise, *DEFB4*, *DEFB6*, *DEFB7*, *DEFB10*, and *LAP* in LPS-stimulated neutrophils at 3 d postpartum were positively associated with serum Ca at 0 d postpartum. Transcripts for *DEFB7*, *DEFB10* and *LAP* also were less abundant in neutrophils from cows with metritis compared with healthy cows. In conclusion, feeding a prepartum negative DCAD to improve postpartum serum Ca resulted in greater neutrophil β -defensin expression, and greater neutrophil β -defensin expression was positively associated with postpartum health.

Key words: dairy cow, calcium, DCAD, neutrophil, β -defensin

INTRODUCTION

Disruptions in energy and mineral homeostasis, combined with demands on the immune system around parturition and increased exposure of the uterus and mammary gland to pathogens, increase risk of postpartum diseases in dairy cows (Chapinal et al., 2011). Two recent meta-analyses indicated that feeding prepartum rations with a negative DCAD compared with positive DCAD was effective at increasing postpartum Ca concentrations in serum, increasing fat-corrected milk yield, and decreasing incidence of retained placenta and metritis (Lean et al., 2019; Santos et al., 2019). We speculate that the decreased incidence of retained placenta and metritis from feeding prepartum negative DCAD results, in part, from improved immune function of cows.

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Postpartum immune suppression is common in dairy cows, and neutrophil dysfunction, in particular, is associated with greater risk of bacterial diseases and uterine disorders (Cai et al., 1994; Hammon et al., 2006). Although several factors contribute to postpartum immune suppression, hypocalcemia is notable because of the critical role of Ca in immune cell activation (Kimura et al., 2006). In neutrophils, immune stimulatory factors, such as LPS, stimulate intracellular Ca via the phospholipase-inositol triphosphate pathway. The intracellular Ca efflux from endoplasmic reticulum stores subsequently initiates influx of extracellular Ca via stromal interaction molecules and Ca release-activated Ca channel proteins in a process known as store-operated Ca entry (SOCE; Clemens and Lowell, 2015). The increased intracellular Ca from SOCE triggers several neutrophil functions, including degranulation, chemotaxis, generation of reactive oxygen species, and phagocytosis (Immler et al., 2018). Accordingly, induced hypocalcemia decreased percentages of neutrophils with oxidative burst and phagocytosis capacity (Martinez et al., 2014), whereas mitigation of hypocalcemia with postpartum calcitriol injection increased percentages of neutrophils with oxidative burst and phagocytosis capacity (Vieira-Neto et al., 2017).

Although neutrophil oxidative burst and phagocytosis capacities are critical and often a focus of immune status during the periparturient period, the neutrophil response to bacterial infection includes production of antimicrobial peptides and formation of extracellular traps, among other key bactericidal mechanisms (Paape et al., 2003; Lippolis et al., 2006). Neutrophil granules are loaded with a class of highly similar β -defensin peptides, which possess antimicrobial activity toward gram-negative and gram-positive bacteria (Selsted et al., 1993). The β -defensins produced by neutrophils include β -defensin (DEFB) 1 to 13, lingual antimicrobial peptide (LAP), and tracheal antimicrobial peptide (TAP). Neutrophil β -defensins are constitutively produced during granulopoiesis and stored in granules. Transcription of several of the bovine β -defensin genes are induced in response to LPS and, to a lesser extent, vitamin D signaling (Merriman et al., 2015; Merriman et al., 2018). Induction of β -defensins in response to LPS occurs via nuclear factor κ B binding of promoter elements of the β -defensin genes (Diamond et al., 2000). Despite the bactericidal activity of neutrophil β -defensins, little is known about neutrophil β -defensin expression in the periparturient period. Silva et al. (2017) showed that *DEFB3*, *DEFB4*, and *DEFB7* transcripts of resting peripheral blood leukocytes were increased at 7 d postpartum compared with prepartum leukocytes. Crookenden et al. (2016) reported that *DEFB1*, *DEFB4*, and *DEFB10* were increased in rest-

ing neutrophils on day of calving compared with 1 wk postpartum. The relationship of postpartum Ca and disease with neutrophil β -defensin expression has not been reported.

Because several neutrophil functions are suppressed postpartum, we hypothesized that neutrophil β -defensin gene expression in response to LPS stimulation would decrease over time after parturition. Furthermore, because prepartum diets with a negative DCAD improve postpartum Ca, we hypothesized that feeding prepartum diets with acidogenic salts also would increase neutrophil β -defensin expression. Therefore, the objectives of this study were to evaluate the expression of neutrophils in periparturient dairy cows in relation to day postpartum, prepartum DCAD, and vitamin D. We also performed a retrospective analysis of the effects of postpartum diseases on neutrophil β -defensin expression, oxidative burst, and phagocytosis.

This paper is a companion to previous reports documenting the effects of DCAD and source of vitamin D on mineral homeostasis, health, and lactation performance (Martinez et al., 2018a, b; Rodney et al., 2018). Feeding prepartum diets with negative DCAD improved postpartum Ca status compared with positive DCAD and decreased incidence of morbidity in the first 30 DIM. Serum Ca was also greater in healthy cows compared with cows with postpartum disease. Feeding calcidiol decreased incidences of both retained placenta and metritis and increased percentage of neutrophils with oxidative burst capacity, compared with feeding cholecalciferol; however, feeding negative DCAD did not affect neutrophil oxidative burst or phagocytosis capacities (Martinez et al., 2018a). Here, we report the expression of β -defensin genes in neutrophils in relation to day relative to calving (d 0 and 3), prepartum dietary treatment, postpartum serum Ca, and incidence of postpartum diseases.

MATERIALS AND METHODS

Animals and Diets

The experiment was conducted at the University of Florida's Dairy Unit (Hague, FL) from February to July 2014, as previously reported (Rodney et al., 2018). All procedures for the care and treatment of animals were approved by the University of Florida's Institutional Animal Care and Use Committee, protocol number 201408331. A total of 79 (28 nulliparous and 51 parous) pregnant, dry Holstein cows were enrolled in the experiment. Only the last 35 parous cows enrolled in the experiment were evaluated for neutrophil β -defensin expression. The experiment followed a randomized complete block design, with cow as the experimental unit.

Treatments were arranged in a factorial with 2 levels of DCAD, positive (+130 mEq/kg; **POS**) or negative (−130 mEq/kg; **NEG**), and 2 sources of vitamin D, vitamin D₃ (cholecalciferol) or 25-hydroxyvitamin D₃ (calcidiol), that were fed at 3 mg for each 11 kg of diet DM. Weekly cohorts of cows were blocked by previous lactation 305 d milk and, within each block, assigned randomly to 1 of the 4 treatments. Cows were moved to a pen with individual feeding gates (Calan Broadbent Feeding System, American Calan Inc., Northwood, NH) at 252 d of gestation, and treatments were initiated at 255 d of gestation.

Neutrophil Cultures

At 0 and 3 d relative to parturition, peripheral blood was collected from the coccygeal vein using BD Vacutainer blood collection tubes with acid citrate dextrose (Becton Dickinson and Company, Franklin Lakes, NJ) and centrifuged at $1,500 \times g$ for 20 min. Neutrophils were isolated from peripheral blood as previously reported (Merriman et al., 2015). For neutrophil isolation, the mononuclear cell layer was removed and, to remove erythrocytes, the neutrophil and erythrocyte layer was mixed with 20 mL cold hypotonic buffer (10.6 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, pH 7.2) for 1 min followed by 10 mL restore solution (10.6 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, 462 mM NaCl, pH 7.2), centrifuged 10 min at $450 \times g$, and repeated until erythrocytes were removed.

Neutrophils were cultured in 1 mL of RPMI 1640 (HyClone Laboratories; Logan, UT) that contained 0.42 mM Ca, 10% FBS (HyClone), and 75 ng/mL 25-hydroxyvitamin D₃ (Sigma-Aldrich, St. Louis, MO). Lipopolysaccharide from *Serratia marcescens* (Sigma-Aldrich) was added to the cultures at a final concentration of 0 or 100 ng/mL of culture media. Neutrophils were cultured in a 5-mL round-bottomed tube at a concentration of 5×10^6 cells/mL in a 37°C humidified 5% CO₂ incubator for 6 h.

Measurement of Gene Expression

Ribonucleic acid was extracted from cell samples using Quick-RNA MiniPrep (ZYMO Research, Irvine, CA), according to manufacturer's instructions, and eluted with 50 µL of RNase-free water. The RNA concentration and quality was determined by measurement of absorbance at 260 nm and 280 nm using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were diluted with RNase-free water to a concentration of 1 µg per 10 µL. The RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies,

Carlsbad, CA) in a 20-µL reaction that contained 10 µL of each RNA sample and 1 µL RNase inhibitor. Reactions were incubated at room temperature 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 (**qPCR**) 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 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. Specificities of primer sets were verified by evaluation of the qPCR product melting curves. In addition, the length and sequence of representative qPCR products were determined with gel electrophoresis (2% agarose, 120 V, 50 min) and Sanger sequencing (performed by the University of Florida Interdisciplinary Center for Biotechnology Research, Gainesville) to verify specificity of each primer set. Primer efficiencies were previously reported (Merriman et al., 2015).

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 protein S9 (*RPS9*) using the formula [$C_t(\text{gene of interest}) - C_t(RPS9) = \Delta C_t$]. The ΔC_t values for each gene were used for statistical analysis as will be described. 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.

Neutrophil Functional Assays

Neutrophil functional assays were performed on all cows ($n = 79$) by in vitro culture with dihydrorhodamine and propidium iodide-labeled *Escherichia coli*, as previously described (Martinez et al., 2012) on −7, 2, and 7 d relative to parturition. Effects of vitamin D (cholecalciferol vs. calcidiol) and DCAD (POS vs. NEG) were previously reported (Martinez et al., 2018a). Fluorescence of rhodamine was used to indicate the percentage of neutrophils with oxidative burst and mean fluorescence intensity (**MFI**) of the oxidative burst. Fluorescence of propidium iodide was used to indicate percentage of neutrophils that phagocytized *E. coli* and MFI of phagocytosis.

Postpartum Diseases and Serum Calcium

Diagnosis of postpartum diseases for cows included in this study was described by Martinez et al. (2018a).

Table 1. Gene primer sequences for quantitative PCR¹

Gene ²	Accession no. ³	Strand	Sequence (5'-3')
<i>DEFB1</i>	NM_001324544	Forward	GTCAGGAATAAGTGATTTTGCAAGC
		Reverse	GCCGGAAACAGATGCCAATC
<i>DEFB3</i>	NM_001282581	Forward	CTCCTCGCACTCCTCTTCCT
		Reverse	GCATCTTCGCCTTCTTCTACCACGA
<i>DEFB4</i>	NM_174775	Forward	TCCTCGCAGTCCTCTTCCT
		Reverse	GGCACAAGAACGGAATACAGA
<i>DEFB5</i>	NM_001130761	Forward	CTCCTCGTGCTCCTCTTCC
		Reverse	ACGGCAGCTTTGAGGATTT
<i>DEFB6</i>	NM_001313991	Forward	CTTCTCTAGCATCAGCCGCA
		Reverse	CGCATCTTCGCCTTCTTTTACCTCC
<i>DEFB7</i>	NM_001102362	Forward	TCTTCCTGGTCCTGTCTGCT
		Reverse	GGTGCCAATCTGTCTCCTGT
<i>DEFB10</i>	NM_001115084	Forward	CTCCTCCTGCTCCTCTTGGT
		Reverse	TGCCAATCTGTCTCATGCGT
<i>DEFB13</i>	NM_001324541	Forward	GCCTCTTCTCCATTGTGAGCC
		Reverse	GACGCCTCCATTCTTACCA
<i>LAP</i>	NM_203435	Forward	ACAGCATGAGGCTCCATCAC
		Reverse	ACCTGATCGGCACACAGATG
<i>TAP</i>	NM_174776	Forward	GCGCTCCTCTTCTGCTGCTG
		Reverse	GCACGTTCTGACTGGGCATTGA
<i>RPS9</i>	NM_001101152	Forward	GTGAGGTCTGGAGGGTCAAA
		Reverse	GGGCATTACCTTCGAACAGA

¹Sequences were validated and published by Merriman et al. (2015).²Primer sequences for transcripts of bovine β -defensins (*DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB6*, *DEFB7*, *DEFB10*, and *DEFB13*), lingual antimicrobial peptide (*LAP*), tracheal antimicrobial peptide (*TAP*), and ribosomal protein S9 (*RPS9*).³Accession numbers from NCBI database: <http://www.ncbi.nlm.nih.gov>.**Table 2.** Effects of LPS stimulation on β -defensin gene expression in neutrophils of postpartum cows¹

Gene ²	Day	$-\Delta C_t^3$		SEM	Fold change ⁴	<i>P</i> -value ⁵		
		NoStim	LPS			LPS	Day	LPS $\times$ Day
<i>DEFB1</i>	0	-4.4	-3.5	0.5	1.8	0.02	<0.01	0.32
	3	-5.3	-5.0		1.2			
<i>DEFB3</i>	0	0.3	2.1	0.6	3.4	<0.01	0.03	<0.01
	3	0.2	0.1		0.9			
<i>DEFB4</i>	0	-2.9	-0.2	0.4	6.4	<0.01	0.03	0.09
	3	-3.1	-2.0		2.3			
<i>DEFB5</i>	0	-5.8	-3.3	0.4	5.7	<0.01	0.19	0.06
	3	-5.6	-4.6		2.0			
<i>DEFB6</i>	0	-1.7	-0.5	0.6	2.4	<0.01	<0.01	0.17
	3	-3.1	-2.7		1.3			
<i>DEFB7</i>	0	0.5	2.1	0.4	2.9	<0.01	0.02	0.04
	3	-0.1	0.1		1.1			
<i>DEFB10</i>	0	0.4	1.2	0.5	2.2	0.02	<0.01	0.05
	3	-0.7	-0.7		1.0			
<i>DEFB13</i>	0	-6.5	-7.7	0.4	0.7	0.08	<0.01	0.32
	3	-7.7	-8.3		0.7			
<i>LAP</i>	0	-1.7	0.1	0.5	3.3	<0.01	0.02	0.09
	3	-2.1	-1.5		1.5			
<i>TAP</i>	0	-8.5	-6.2	0.5	5.1	<0.01	0.72	0.11
	3	-8.2	-6.8		2.7			

¹Peripheral blood neutrophils collected from cows ($n = 35$) at d 0 and 3 relative to parturition were stimulated with LPS or no LPS (NoStim).²Gene abbreviations: bovine β -defensins (*DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB6*, *DEFB7*, *DEFB10*, and *DEFB13*), lingual antimicrobial peptide (*LAP*), and tracheal antimicrobial peptide (*TAP*).³Values represent LSM and SEM of $-\Delta C_t [C_{t(RPS9)} - C_{t(target)}]$. Relative transcript abundance is calculated by the transformation: $2^{-\Delta C_t}$. C_t = threshold cycle; RPS9 = ribosomal protein S9.⁴Represents fold change within day in transcript abundance in response to LPS treatment.⁵Significance for effects of LPS, day, and interaction between LPS and day (LPS $\times$ Day).

Briefly, cows were observed daily for disease until 30 DIM, with complete physical examination at 4, 7, and 12 DIM. Retained placenta (RP) was diagnosed based on failure to expel fetal membranes within 12 h

postpartum. Metritis was diagnosed based on presence of flaccid enlarged uterus and watery, fetid, and discolored discharge. Morbidity was defined as incidence of any clinical disease in the first 30 DIM. Subclinical

Figure 1. Peripheral blood neutrophils collected from cows ($n = 35$) at d 0 and 3 relative to parturition were stimulated with LPS or no LPS (NoStim). Data represent LSM $\pm$ SE of expression of bovine β -defensin (*DEFB3*, *DEFB4*, *DEFB6*, *DEFB7*, and *DEFB10*) and lingual antimicrobial peptide (*LAP*) genes normalized to ribosomal protein S9 (*RPS9*) gene expression. Effects of LPS and interaction between LPS and day (LPS $\times$ Day) for each gene are indicated on the respective plot. Effect of day was significant ($P < 0.05$) for each gene. ^{a-c}Means with different letters are significantly different, $P < 0.05$.

hypocalcemia (SCH) was diagnosed as postpartum serum total Ca < 2.0 mM. Serum total Ca was measured using atomic absorption spectrophotometry (AAnalyst 200, Perkin Elmer, Waltham, MA).

Statistical Analysis

Gene expression data were analyzed using the ΔC_t values for each gene. Neutrophil function data (oxidative burst and phagocytosis) and gene expression data were evaluated for normal distribution and analyzed with generalized linear mixed models (Proc Glimmix) using SAS (version 9.4, SAS Inc., Cary, NC). The Kenward-Roger method was used to calculate the denominator degrees of freedom to approximate the F -tests in the statistical models. The initial model for gene expression data included fixed effects of dietary vitamin D treatment (cholecalciferol vs. calcidiol), DCAD (POS vs. NEG), LPS stimulation (0 vs. 100 ng/mL in culture), day postpartum (0 vs. 3 d), and cow nested within block and dietary treatment as random effect. The effect of vitamin D treatment was not significant and was excluded from further analyses. The analysis for effect of DCAD and interaction between DCAD and day only included data from LPS-stimulated neutrophils. Additional analyses were performed using only

data from LPS-stimulated samples to test for fixed effects of postpartum disease (SCH, metritis, RP, and all morbidity) and serum Ca (0 d and 3 d). The ΔC_t values were used as the response variables and transformed as described earlier to report the relative abundance of each β -defensin gene.

Effects of DCAD and vitamin D on neutrophil oxidative burst and phagocytosis were previously reported (Martinez et al., 2018a). Retrospective analyses for effects of postpartum SCH and metritis on neutrophil oxidative burst and phagocytosis were analyzed with a model that included fixed effects of disease, day, interaction between disease and day, and random effects of cow. Pearson correlations for serum total Ca and gene expression were performed with Prism 6 (GraphPad Software Inc., San Diego, CA) using $-\Delta C_t$ values as the measure of gene expression. For all data, differences were considered significant at $P \leq 0.05$.

RESULTS

Effects of LPS Stimulation and Day Postpartum on Neutrophil β -Defensins

Treatment of neutrophils with LPS increased expression of each β -defensin gene except *DEFB13*, which

Table 3. Effects of prepartum DCAD on neutrophil β -defensin gene expression¹

Gene ²	Day	Transcript abundance, ³ $-\Delta C_t$			P-value ⁴		
		POS	NEG	SEM	DCAD	Day	DCAD $\times$ Day
<i>DEFB1</i>	0	-3.7	-3.3	0.6	0.31	<0.01	0.48
	3	-5.6	-4.5				
<i>DEFB3</i>	0	1.5	3.0	0.8	0.32	<0.01	0.42
	3	-0.2	0.3				
<i>DEFB4</i>	0	-1.2	0.8	0.9	0.04	0.01	0.69
	3	-3.2	-0.7				
<i>DEFB5</i>	0	-3.6	-2.8	0.7	0.14	0.06	0.64
	3	-5.4	-3.9				
<i>DEFB6</i>	0	-0.5	-0.3	0.6	0.36	<0.01	0.46
	3	-3.3	-2.2				
<i>DEFB7</i>	0	1.5	3.0	0.7	0.08	<0.01	0.92
	3	-0.6	0.8				
<i>DEFB10</i>	0	1.0	2.6	0.8	0.11	<0.01	0.77
	3	-1.3	-0.1				
<i>DEFB13</i>	0	-7.1	-6.8	0.9	0.64	0.02	0.64
	3	-8.7	-8.0				
<i>LAP</i>	0	-0.0	0.4	0.6	0.39	<0.01	0.85
	3	-1.8	-1.2				
<i>TAP</i>	0	-5.5	-6.5	0.7	0.42	0.05	0.48
	3	-7.0	-7.2				

¹Peripheral blood neutrophils collected at d 0 and 3 relative to parturition from cows fed prepartum ration with positive (POS, n = 16) or negative (NEG, n = 19) DCAD. Neutrophils were stimulated with lipopolysaccharide and analyzed for gene expression.

²Gene abbreviations: bovine β -defensins (*DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB6*, *DEFB7*, *DEFB10*, and *DEFB13*), lingual antimicrobial peptide (*LAP*), and tracheal antimicrobial peptide (*TAP*).

³Values represent LSM and SEM of $-\Delta C_t [C_{t(RPS9)} - C_{t(target)}]$. Relative transcript abundance is calculated by the transformation: $2^{-\Delta C_t}$. C_t = threshold cycle; RPS9 = ribosomal protein S9.

⁴Significance for effects of prepartum DCAD (POS vs. NEG), day, and interaction between DCAD and day (DCAD $\times$ Day).

tended to be decreased by LPS stimulation (Table 2). Except for *DEFB5* and *TAP*, each of the β -defensins also were more abundant ($P < 0.05$) in neutrophils on day of calving compared with 3 d postpartum. Further-

more, an interaction between LPS and day postpartum was significant for several of the β -defensins, such that expression did not increase in response to LPS on d 3 postpartum, in contrast to increased expression in

Figure 2. Peripheral blood neutrophils collected at d 0 and 3 relative to parturition from cows fed prepartum ration with positive (POS, $n = 16$) or negative (NEG, $n = 19$) DCAD. Data represent LSM $\pm$ SE of expression of bovine β -defensin (*DEFB3*, *DEFB4*, *DEFB6*, *DEFB7*, and *DEFB10*) and lingual antimicrobial peptide (*LAP*) genes normalized to ribosomal protein S9 (*RPS9*) gene expression in neutrophils stimulated with LPS. Effects of DCAD and interaction between DCAD and day (DCAD $\times$ Day) for each gene are indicated on the respective plot. Effect of day was significant ($P < 0.05$) for each gene. ^{a-c}Means with different letters are significantly different, $P < 0.05$.

response to LPS on day of calving (Table 2 and Figure 1). The LPS-stimulated neutrophils of NEG cows had greater *DEFB4* ($P = 0.04$) and *DEFB7* ($P = 0.08$) expression compared with POS cows (Table 3 and Figure 2). Parturition vitamin D treatment did not affect ($P > 0.1$) any of the β -defensins in neutrophils, nor did we find an interaction ($P > 0.1$) between vitamin D and DCAD treatments for any of the neutrophil β -defensins (data not shown).

Neutrophil β -defensins were evaluated retrospectively for effects of Ca status (Table 4). Cows that experienced postpartum SCH (serum Ca < 2.0 mM) had decreased *DEFB3* ($P = 0.06$), *DEFB4* ($P = 0.02$), *DEFB5* ($P = 0.03$), *DEFB7* ($P = 0.04$), *DEFB10* ($P = 0.02$), and *LAP* ($P = 0.02$) expression (Table 4). The interactions between day and incidence of SCH were also significant ($P < 0.05$) for *DEFB4*, *DEFB6*, *DEFB7*, *DEFB10*, and *LAP*, such that at 0 d expression did not differ because of SCH, but at d 3 postpartum the β -defensin transcripts were less abundant in cows with SCH compared with normocalcemic cows (Table 4, Figure 3).

Neutrophil β -defensin in LPS-stimulated neutrophils at 0 and 3 d was plotted against serum total Ca at 0 and 3 d to examine for correlations between neutrophil β -defensin expression and serum Ca. Expression of the

most abundant neutrophil β -defensin, *DEFB7*, plotted against serum total Ca is shown in Figure 4, as a representation. None of the β -defensins at 0 d were correlated with serum Ca at 0 d (Figure 4A). Neutrophil *DEFB7* at 0 d tended to be correlated ($P = 0.06$, $r = 0.34$) with serum Ca at 3 d (Figure 4B). Neutrophil *DEFB10* at 0 d also was correlated ($P = 0.04$, $r = 0.36$) with serum Ca at 3 d, but the other β -defensins at 0 d were not correlated with serum Ca at 3 d. In contrast, *DEFB6*, *DEFB7*, *DEFB10*, and *LAP* transcripts at 3 d were positively correlated ($P < 0.05$) with serum Ca at 0 d (Figure 4C); however, neither of those transcripts at 3 d were correlated with serum Ca at 3 d (Figure 4D).

The effects of postpartum disease (RP, metritis, and all morbidity) on each β -defensin in LPS-stimulated neutrophils also were evaluated retrospectively (Table 5). Neutrophils of cows with RP had less ($P = 0.05$) *DEFB7* at 3 d postpartum compared with cows with no RP, but *DEFB7* did not differ at 0 d because of RP (Figure 5A). An interaction between RP and day also was significant for *LAP* (Figure 5A). Neutrophils of cows with metritis also had less *DEFB7* ($P = 0.05$) and *LAP* ($P = 0.02$) compared with cows that did not have metritis (Figure 5B). Similarly, neutrophils of

Table 4. Effects of postpartum Ca status on neutrophil β -defensin gene expression

Gene ¹	Day	Transcripts, ² $-\Delta C_t$			P-value ³		Correlation ⁴			
		No	Yes	SEM	SCH	SCH $\times$ Day	Ca, d 0		Ca, d 3	
							r	P-value	r	P-value
<i>DEFB1</i>	0	-3.3	-3.6	0.6	0.04	0.03	0.15	0.40	0.33	0.06
	3	-3.5	-6.1				0.33	0.06	0.22	0.22
<i>DEFB3</i>	0	2.7	1.9	0.8	0.07	0.10	0.07	0.69	0.28	0.11
	3	1.6	-1.1				0.23	0.19	0.16	0.35
<i>DEFB4</i>	0	0.3	-0.5	0.8	0.02	0.02	-0.10	0.60	0.02	0.89
	3	0.4	-3.6				0.21	0.24	0.02	0.90
<i>DEFB5</i>	0	-3.0	-3.3	0.7	0.03	0.06	-0.07	0.69	0.20	0.26
	3	-2.9	-5.8				0.25	0.15	0.05	0.77
<i>DEFB6</i>	0	-0.5	-0.4	0.6	0.11	0.01	-0.11	0.56	0.13	0.48
	3	-1.3	-3.8				0.43	0.01	0.12	0.51
<i>DEFB7</i>	0	2.4	2.1	0.7	0.04	0.02	-0.03	0.88	0.34	0.06
	3	1.9	-1.2				0.39	0.02	0.20	0.25
<i>DEFB10</i>	0	2.1	1.6	0.7	0.02	0.02	0.01	0.94	0.36	0.04
	3	1.3	2.1				0.35	0.04	0.11	0.52
<i>DEFB13</i>	0	-7.1	-6.9	0.9	0.53	0.12	0.02	0.93	0.18	0.31
	3	-7.4	-9.0				0.11	0.53	0.19	0.28
<i>LAP</i>	0	0.3	0.1	0.6	0.02	0.02	0.02	0.92	0.27	0.14
	3	0.1	-2.6				0.44	0.01	0.24	0.16
<i>TAP</i>	0	—	—	—	—	—	-0.05	0.77	0.23	0.21
	3	—	—				0.38	0.03	0.35	0.04

¹Gene abbreviations: bovine β -defensins (*DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB6*, *DEFB7*, *DEFB10*, and *DEFB13*), lingual antimicrobial peptide (*LAP*). The model for tracheal antimicrobial peptide (*TAP*) did not solve for each condition and was omitted.

²Cows were retrospectively grouped according to no incidence (No) of postpartum subclinical hypocalcemia (SCH) or incidence of SCH (Yes). Values represent LSM and SEM of $-\Delta C_t$ [$C_{t(RPS9)} - C_{t(target)}$] of lipopolysaccharide-stimulated neutrophils. Relative transcript abundance is calculated by the transformation: $2^{-\Delta C_t}$. C_t = threshold cycle; RPS9 = ribosomal protein S9.

³Significance for effects of postpartum SCH, and interaction between SCH and Day (SCH $\times$ Day).

⁴Pearson coefficients and significance of correlations between $-\Delta C_t$ of each gene on d 0 with serum total Ca on d 0 or 3 relative to parturition.

cows with any postpartum clinical disease tended to have less ($P < 0.10$) *DEFB7* and *LAP* compared with healthy cows (Table 5).

Finally, neutrophil oxidative burst and phagocytosis capacities, which were measured in all cows ($n = 79$),

were retrospectively analyzed according to incidences of SCH (Figure 6) and metritis (Figure 7). Cows with SCH tended ($P = 0.08$) to have a decreased percentage of neutrophils with oxidative burst, compared with cows that did not experience SCH (Figure 6B). Intensi-

Figure 3. Peripheral blood neutrophils collected at d 0 and 3 relative to parturition and stimulated with LPS. Cows were retrospectively diagnosed with normal Ca (Norm, $n = 15$) or subclinical hypocalcemia (SCH, $n = 20$) on the basis of serum total Ca < 2.0 mM. Data represent LSM $\pm$ SE of expression of bovine β -defensin (*DEFB3*, *DEFB4*, *DEFB6*, *DEFB7*, and *DEFB10*) and lingual antimicrobial peptide (*LAP*) genes normalized to ribosomal protein S9 (*RPS9*) gene expression in neutrophils stimulated with LPS. Effects of SCH and interaction between SCH and day (SCH $\times$ Day) for each gene are indicated on the respective plots. Effect of day was significant ($P < 0.05$) for each gene. ^{a,b}Means with different letters are significantly different, $P < 0.05$.

ties of oxidative burst and phagocytosis and percentage of neutrophils with phagocytosis capacity were not affected by incidence of SCH (Figure 6). In contrast, neutrophils of cows with metritis had higher ($P = 0.003$) intensities of oxidative burst (Figure 7A) but not percentages of neutrophils with oxidative burst or phagocytosis or phagocytosis intensity, compared with cows that did not experience metritis (Figure 7B–D).

DISCUSSION

Mitigation of postpartum hypocalcemia in dairy cows by feeding prepartum acidogenic salts to create a negative DCAD improves health and production of dairy cows (Lean et al., 2019; Santos et al., 2019). The

negative effect of hypocalcemia on immune cell activation in dairy cows is well established (Kimura et al., 2006; Martinez et al., 2014), and we speculate that the improved health and production from feeding prepartum acidogenic salts results in part from greater Ca availability for immune cell activation. Although cows fed a negative DCAD did not have improved neutrophil oxidative burst or phagocytosis (Martinez et al., 2018a), we report that feeding a negative DCAD diet improved expression of *DEFB4* and *DEFB7* in LPS-stimulated neutrophils of postpartum cows. Perhaps more striking, we observed, cows that experienced SCH had decreased expression of several β -defensin genes at 3 d postpartum, compared with cows that did not experience SCH, and β -defensin gene expression at 3 d

Figure 4. Peripheral blood neutrophils collected from cows ($n = 35$) at d 0 and 3 relative to parturition and stimulated with LPS. (A) The $-\Delta C_t$ of β -defensin 7 (*DEFB7*) on d 0 vs. serum total Ca on d 0. (B) The $-\Delta C_t$ of *DEFB7* on d 0 vs. serum total Ca on d 3. (C) The $-\Delta C_t$ of *DEFB7* on d 3 vs. serum total Ca on d 0. (D) The $-\Delta C_t$ of *DEFB7* on d 3 vs. serum total Ca on d 3. Pearson correlation coefficients and significance of correlations are indicated on the respective plots.

postpartum was consistently associated with serum Ca on day of calving. Although the contribution of neutrophil β -defensins to disease resistance cannot be determined from this study, our data strengthen evidence that mitigation of hypocalcemia improves the ability of neutrophils to respond to bacterial pathogens.

Induction of bovine neutrophil β -defensins in response to LPS likely occurs through toll-like receptor 4 (TLR4) activation of NF- κ B signaling (Diamond et al., 2000). Here, we observed that in vitro LPS stimulation was a potent inducer of β -defensin expression in neutrophils, similar to previous experiments with bovine neutrophils and mammary epithelial cells (Merriman et al., 2015). Merriman et al. (2015) also reported that neutrophil β -defensin expression was not affected by vitamin D signaling, in contrast to monocytes; thus it is not surprising that prepartum vitamin D source (cholecalciferol vs. calcidiol) fed to cows in this experiment did not affect neutrophil β -defensin expression. Similar to neutrophil gene expression reported by Crookenden et al. (2016), we found that day relative to calving affected all β -defensins except *DEFB5* and *TAP*, except we also observed that several of the most abundant β -defensins in neutrophils were less responsive to LPS stimulation at 3 d postpartum than on day of calving. The decreased responsiveness of neutrophils to LPS at 3 d postpartum is consistent with immunosuppression that occurs after parturition.

Decreased induction of neutrophil β -defensins in response to LPS stimulation may occur through a combination of stressors associated with metabolic, immune, and hormonal perturbations that occur in the transition from pregnancy to lactation (Sordillo, 2016). Our data show that LPS-stimulated neutrophils

of cows with SCH had decreased β -defensin expression at 3 d postpartum compared with cows that did not experience SCH, and, more specifically, serum Ca on day of calving was consistently correlated with β -defensin expression at 3 d postpartum. If LPS does indeed signal via TLR4 and NF- κ B pathways to induce β -defensin expression, a direct role for Ca is unclear. Although intracellular Ca signaling via SOCE is integral for neutrophil oxidative burst and phagocytosis, we are unaware of a definitive role for Ca signaling in TLR4 activation of NF- κ B. Conejeros et al. (2015) reported that synthetic TLR2 and TLR1 ligand, PAM₃CSK₄, but not ultrapure LPS, stimulated SOCE in bovine neutrophils. Clemens et al. (2017) reported that SOCE activated the NF- κ B pathway in murine neutrophils, which increased cytokine gene expression and protein production. Thus, evidence exists that SOCE can influence NF- κ B signaling in neutrophils, so it is plausible that SOCE could affect NF- κ B induction of neutrophil β -defensin expression. If so, diminished SOCE in neutrophils from cows that experienced SCH explains the decreased β -defensin expression in response to LPS.

The consistent correlation of several neutrophil β -defensins at 3 d postpartum with serum Ca at day of calving makes sense, considering the dynamics of neutrophil development. Although circulating neutrophils have a high rate of turnover (6 to 8 h), the process of granulopoiesis takes about 5 d (Summers et al., 2010). In addition, approximately 50% of total blood neutrophils exist as marginated pools within tissue vasculature, which can have a considerably longer lifespan. Thus, developing neutrophils in the bone marrow and marginated neutrophils at day of calving, the low point of serum Ca, were likely the neutrophils harvested and

Table 5. Effects of postpartum disease on neutrophil β -defensin gene expression¹

Gene ²	P-value ³					
	RP	RP $\times$ Day	Metr	Metr $\times$ Day	Morb	Morb $\times$ Day
<i>DEFB1</i>	0.18	0.32	0.79	0.67	0.63	0.46
<i>DEFB3</i>	0.28	0.34	0.21	0.70	0.78	0.14
<i>DEFB4</i>	0.65	0.07	0.92	0.34	0.33	0.54
<i>DEFB5</i>	0.31	0.05	0.18	0.24	0.38	0.21
<i>DEFB6</i>	0.92	0.14	0.54	0.25	0.32	0.24
<i>DEFB7</i>	0.05	0.05	0.05	0.16	0.34	0.08
<i>DEFB10</i>	0.14	0.14	0.08	0.41	0.67	0.10
<i>DEFB13</i>	0.98	0.38	0.85	0.81	0.26	0.51
<i>LAP</i>	0.12	0.06	0.02	0.13	0.16	0.06

¹Neutrophils collected on d 0 and 3 relative to parturition were stimulated with LPS. Cows were retrospectively grouped according to incidence of postpartum diseases.

²Gene abbreviations: bovine β -defensins (*DEFB1*, *DEFB3*, *DEFB4*, *DEFB5*, *DEFB6*, *DEFB7*, *DEFB10*, and *DEFB13*), lingual antimicrobial peptide (*LAP*). The model for tracheal antimicrobial peptide did not solve for each condition and was omitted.

³Significance for effects of retained placenta (RP), interaction between RP and day (RP $\times$ Day), metritis (Metr), interaction between metritis and day (Metr $\times$ Day), morbidity in the first 30 d postpartum (Morb), and interaction between morbidity and day (Morb $\times$ Day).

Figure 5. Peripheral blood neutrophils collected at d 0 and 3 relative to parturition and stimulated with lipopolysaccharide. (A) Cows were observed for retained placenta and retrospectively grouped according to incidence of retained placenta (RP, $n = 7$) or no RP (Healthy, $n = 28$). (B) Cows were observed for metritis and retrospectively grouped according to incidence of metritis (Metr, $n = 9$) or no metritis (Healthy, $n = 26$). Data represent LSM $\pm$ SE of expression of bovine β -defensin (*DEFB7*) and lingual antimicrobial peptide (*LAP*) genes normalized to ribosomal protein S9 (*RPS9*). Effects of RP, interaction between RP and day (RP $\times$ Day), Metr, and interaction between Metr and day (Metr $\times$ Day) for each gene are indicated on the respective plots. ^{a,b}Means with different letters are significantly different, $P < 0.05$.

tested at 3 d postpartum. Furthermore, the extracellular Ca concentrations at time of activation seem to be less critical than Ca stores in endoplasmic reticulum compartments of neutrophils for SOCE of Ca from the extracellular space. Martinez et al. (2014) reported that percentages of neutrophils with oxidative burst and phagocytosis capacity were diminished by induced hypocalcemia at least 48 h after serum Ca returned to normal, similar to the delayed effect we observed here. Neutrophils from cows with induced hypocalcemia had similar cytosolic Ca compared with control cows before stimulation but decreased cytosolic Ca compared with control cows after stimulation. The effect of induced hypocalcemia on cytosolic Ca concentration and neu-

trophil function occurred regardless of performing the assays in media with low or normal Ca concentrations. Altogether, we take this information to mean that hypocalcemia results in impaired SOCE in neutrophils, which manifests for several days after hypocalcemia because of decreased endoplasmic reticulum stores of Ca in neutrophils. This coincides with reports that treatment of hypocalcemia with an oral Ca bolus, which results in a transient recovery of serum Ca, did not improve neutrophil functions (Milteneburg et al., 2018), whereas a sustained increase of Ca from calcitriol treatment or prevention of hypocalcemia from feeding a prepartum negative DCAD improved neutrophil function (Vieira-Neto et al., 2017).

Figure 6. Peripheral blood neutrophils collected at d 0 and 3 relative to parturition and stimulated with LPS. Cows were retrospectively diagnosed with normal Ca (Norm, $n = 48$) or subclinical hypocalcemia (SCH, $n = 31$) on the basis of serum total Ca <2.0 mM. Data represent LSM $\pm$ SE of oxidative burst intensity of neutrophils as measured by the log median fluorescence intensity (MFI) of rhodamine 123 (A), percentage of neutrophils with oxidative burst (B), neutrophil phagocytosis as measured by MFI of propidium iodide (C), and percentage of phagocytic neutrophils (D). Effects of SCH and interaction between SCH and day (SCH $\times$ Day) are indicated on the respective plots.

Postpartum immune dysfunction is associated with increased risk of postpartum diseases, such as metritis (Galvão et al., 2012; Bromfield et al., 2018). We observed that *DEFB7*, *DEFB10*, and *LAP* transcripts in LPS-stimulated neutrophils were decreased in cows that experienced metritis compared with cows that did not. Although we measured β -defensin gene expression, not bactericidal activity, and the decreased β -defensin expression may merely be associated with or represent impaired immunity, we hypothesize that decreased neutrophil β -defensin expression contributes to greater risk of postpartum diseases. Even though neutrophil β -defensins are only 1 component of the neutrophil's defense against bacterial pathogens, they are highly

expressed in the endometrium during endometritis, and they possess potent bactericidal activity (Selsted et al., 1993; Walker et al., 2015). Our data indicate the need to determine the degree to which hypocalcemia affects abundance of β -defensin peptides and other antimicrobial peptides in tissues during bacterial infection.

In contrast to neutrophil β -defensin expression, the intensity of neutrophil oxidative burst was higher in cows that developed metritis compared with cows that did not. We find the observation of greater neutrophil oxidative burst in metritis cows interesting, as others have documented that neutrophils of cows with postpartum diseases had decreased functional capacities (Cai et al., 1994; Kimura et al., 2002; Hammon et al.,

Figure 7. Peripheral blood neutrophils collected at d 0 and 3 relative to parturition and stimulated with lipopolysaccharide. Cows were examined for metritis on 4, 7, and 12 DIM and retrospectively grouped according to incidence of metritis (Metr, $n = 27$) or no metritis (Healthy, $n = 52$). Data represent LSM $\pm$ SE of oxidative burst intensity of neutrophils as measured by the log median fluorescence intensity (MFI) of rhodamine 123 (A), percentage of neutrophils with oxidative burst (B), neutrophil phagocytosis as measured by MFI of propidium iodide (C), and percentage of phagocytic neutrophils (D). Effects of Metr and interaction between Metr and day (Metr $\times$ Day) are indicated on the respective plots.

2006). Neutrophil oxidative burst and phagocytosis are commonly used as indices of immune status in transition cow experiments. Oxidative burst and phagocytosis certainly play a critical role in clearance of bacterial pathogens (Paape et al., 2003), and the percentages of neutrophils with oxidative burst and phagocytosis capacity are consistently associated with postpartum diseases; however, a greater intensity of neutrophil oxidative burst, as with most immune parameters, β -defensins included, should not be readily interpreted as indicative of improved disease resistance. Instead, the greater oxidative burst intensity of neutrophils from metritis cows compared with healthy cows may represent a greater propensity toward oxidative damage of tissues that contributes to pathogenesis of diseases such as mastitis and metritis (Sordillo, 2018). As with the β -defensins, our data indicate the need to assess the role of neutrophil oxidative burst capacity on oxidative damage of tissues and resolution of disease.

CONCLUSIONS

Neutrophil β -defensin expression decreased at 3 d postpartum compared with day of calving. The decreased neutrophil β -defensin expression at 3 d postpartum was associated with postpartum SCH and was mitigated, to some extent, by feeding a prepartum negative DCAD. Decreased neutrophil β -defensin expression was also associated with postpartum RP and metritis, in contrast to intensity of neutrophil oxidative burst, which was increased in cows that developed metritis. Our data suggest a potential role for neutrophil β -defensins in the positive benefits to postpartum health and production of dairy cows from feeding prepartum rations with negative DCAD.

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