Systemic and local responses of cytokines and tissue histology following intramammary lipopolysaccharide challenge in dairy cows

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ABSTRACT

During bovine mastitis, immune responses include the release of cytokines and the recruitment of leukocytes, resulting in profound structural and functional changes in the mammary gland. Our aims were to delineate systemic and local cytokine responses and to quantify histological changes in the mammary tissue of lactating cows after acute intramammary lipopolysaccharide (LPS) challenge. Ten multiparous dairy cows were paired to either treatment (TRT) or control (CON) groups. For TRT cows, one side of the udder was randomly assigned to receive treatment with LPS (50 µg in 10 mL of saline, TL) into both the front and rear quarters; the contralateral quarters received saline (10 mL). Udder-halves of CON cows were similarly assigned randomly to receive either saline (10 mL, CS) or no infusion (untreated). Temporal changes in the concentrations of 15 cytokines in the blood (0, 3, 6, 12, and 24 h relative to the LPS infusion) and in mammary tissue (0, 3, and 12 h) were determined, as were concomitant changes in mammary histology. The cytokines IL-6, IL-10, MCP-1, and MIP-1β showed a systemic response as their concentrations were significantly different in the plasma of TRT cows as compared with CON cows after LPS challenge. The cytokines IL-1α, IL-1β, IL-6, IL-8, IL-17A, IL-36RA, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and VEGF-A showed a local response in TL glands, and 8 cytokines, IL-1β, IL-6, IL-17A, IL-36RA, IP-10, MIP-1β, and MIP-1α showed systemic changes in the nonchallenged mammary glands adjacent to LPS-infused glands. Endotoxin challenge evoked changes in the histology of mammary tissue that included a 5.2- and 7.2-fold increases in the number of neutrophils in alveolar lumens at 3 h and 12 h, respectively. In summary, LPS challenge induced specific local and systemic responses in cytokine induction and elicited neutrophil infiltration in bovine mammary tissue.

Key words: histological change, inflammation mediator, lipopolysaccharide, mammary gland, mastitis

INTRODUCTION

Mastitis is typically the most common and most costly disease in dairy cows across the globe. It not only causes a reduction in the quality and quantity of milk produced but may also cause irreparable mammary tissue damage. As one of a wide array of virulence factors that are associated with mastitis-causing pathogens, LPS is recognized by the mammary immune system, which responds by secreting cytokines (Johnson et al., 2018; Naylor et al., 2020), consequently triggering inflammation (Akers and Nickerson, 2011). Use of LPS to model the host response and pathologies related to gram-negative bacterial infection in livestock has been applied widely for research purposes.

Lipopolysaccharides (also known as endotoxins) are structural lipoglycans found in the outer membrane of gram-negative bacteria. They are the most potent pathogen-associated molecular patterns that produce inflammatory responses (Mogensen, 2009). When gram-negative bacteria enter the mammary gland through the teat canal, they encounter mammary epithelial cells (MEC) and immune cells, which recognize LPS and release cytokines. Cytokines are small protein molecules that orchestrate communication with immune cells to regulate inflammatory responses and the return to homeostasis afterward. The signals transmitted by cytokines may be stimulatory or inhibitory, thereby eliciting balanced immune responses. Examples of cytokines include both proinflammatory (e.g., IFNG, IL1A/B, IL6, and TNFA) and anti-inflammatory (e.g., IL10 and IL36RA) cytokines, chemokines that attract immune cells including neutrophils (e.g., IL1A/1B, IL8/CXCL8, IL17A, and TNFA), eosinophils (e.g., IL4), and T-cells (e.g., CCL3/MIP1A and CCL4/MIP1B), and growth factors (e.g., VEGF).
and CCL2) and proinflammatory cytokines (IL1B, IL6, and TNFA) are known to be released by MEC to attract neutrophils from circulation (Petzl et al., 2012; Védrine et al., 2018). Thus, MEC are a crucial component of the first line of defense provided by the innate immune system of the mammary gland.

The histological appearance of lactating bovine mammary glands is characterized by well-developed mammary parenchyma with numerous alveoli comprised of secretory epithelial cells surrounding large alveolar lumens, and only a scant amount of connective tissue (Holland and Holland, 2005; Akers et al., 2006). During mastitis, the breakdown of tight junction protein complexes between MEC leads to the disruption of alveolar structures and the blood-milk barrier. A disruption of MEC integrity and the sloughing of cells into alveolar lumens are hallmarks of induced apoptosis, which often occurs during mastitis insult. On one hand, neutrophil influx in the mammary tissue combats inflammation, while in contrast, it impairs milk synthesis and secretory functions of MEC (Zhao and Lacasse, 2008; Akers and Nickerson, 2011). Indeed, 24 h after inoculation with 500 to 5,000 cfu of Staphylococcus aureus, the MEC secretory area and open luminal area of bovine mammary glands were dramatically decreased (Akers and Nickerson, 2011). However, an important advantage of using LPS instead of live bacterial inoculum is the ability to induce a relatively predictable inflammatory response, without the complicating effects of pathogen proliferation and the accompanying destruction of MEC. Thus, it is possible to distinguish between systemic and local mammary responses to mastitis.

In our recent companion study, acute intramammary LPS challenge resulted in a large spike in SCC, but only in the milk of LPS-injected glands, whereas both challenged and nonchallenged glands suffered marked declines (20%–30%) in milk yield, demonstrating a significant systemic effect on milk yield and compositions (Shangraw et al., 2020). To delineate the possible factors associated with these local and systemic changes, the present study investigated both systemic and tissue-level changes in the concentrations of 15 cytokines after intramammary LPS challenge and analyzed the local effects of LPS on mammary histomorphology. In addition, the time-dependent effects of endotoxemia on cytostructure in the mammary gland were also investigated.

**MATERIALS AND METHODS**

**Animal Treatment and Sample Collection**

All use of animals was approved by the Institutional Animal Care and Use Committees at the University of Vermont (IACUC #17–028) and the University of Missouri (protocol no. 9283; Shangraw et al., 2020). Ten multiparous, lactating cows were assigned to pairs based on breed (8 Holstein-Friesian and 2 Jersey × Holstein) and milk yield (22.3–37.7 kg/d; n = 5). One cow of each pair was randomly assigned to either the treatment (TRT) or control (CON) group. All cows included in the study were clinically healthy and had cow-level SCC <174,000. Cows were milked twice daily at ~0600 and 1800 h. The milk samples were taken from all quarters of eligible cows on d −14 and −8 before the experiment using aseptic milking techniques and tested for mastitis pathogens by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF; Barreiro et al., 2010). Alludder quarters were negative for major mastitis pathogens. The experiment was performed at the University of Missouri, Foremost Dairy Farm (Columbia, MO).

**Intramammary Endotoxin Treatment.** Right after the morning milk and first udder biopsy (see below), the front and rear udder quarters of one randomly chosen udder-half (left or right side) were each infused via the teat canal with 50 µg of LPS (Serotype O55:B5; Sigma-Aldrich, St. Louis, MO) in 10 mL of sterile saline in TRT cows. This udder-half was designated (TL). The contralateral side received 10 mL of saline per quarter and was designated (TS). For CON cows, each quarter corresponding to a TL quarter of the paired cows received 10 mL of saline; these quarters were designated as the glands that received saline (CS). The contralateral side received no infusion and remained untreated (CU).

**Blood Collection.** Blood samples were collected from the tail (coccygeal) vein into EDTA-containing Vacutainer tubes right before LPS challenge (0 h) and at 3, 6, 12, and 24 h after challenge. Blood was processed as soon as possible after collection (<5 min). Blood was centrifuged at 2,200 × g for 15 min at 4°C, supernatants were collected, and aliquots of plasma were stored at −80°C until further analysis.

**Mammary Biopsy.** Mammary biopsies were obtained as described (Wall et al., 2013; Shangraw et al., 2020). Briefly, on the day of the biopsy, cows were restrained in a chute and lightly sedated by intravenous injection of xylazine (20–30 µg/kg BW; Akorn Inc., Lake Forest, IL). Chosen biopsy sites, on rear quarters only, were shaved and aseptically prepared using 70% alcohol scrubs, then local anesthesia (1.5–3.0 mL of lidocaine) was administered in a line-block above the incision site. Biopsies (500–800 mg of tissue) were obtained at 0, 3, and 12 h, relative to LPS challenge, from both hind quarters of all cows. Subsamples of tissue were either snap frozen in liquid nitrogen and stored at −80°C until protein extraction or processed for histol-
Bovine Cytokine Array

For plasma and mammary tissue homogenates, the concentrations of 15 cytokines (interferon gamma [IFN-γ], interleukin 1α [IL-1α], interleukin 1β [IL-1β], interleukin 4 [IL-4], interleukin 6 [IL-6], interleukin 8 [IL-8], interleukin 10 [IL-10], interleukin 17A [IL-17A], Interleukin 36 receptor antagonist [IL-36RA], interferon gamma-induced protein 10 [IP-10]/C-X-C motif chemokine ligand 10 [CXCL10], monocyte chemotactrant protein 1 [MCP-1]/chemokine [C-C motif] ligand 2 [CCL2], macrophage inflammatory protein α [MIP-1α or CCL3], macrophage inflammatory protein β [MIP-1β or CCL4], tumor necrosis factor α [TNF-α], and vascular epithelial growth factor [VEGF-A]) were measured simultaneously using the MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel 1–Immunology Multiplex Assay (BCYT1–33K, Millipore Sigma, Burlington, MA) performed by Eve Technologies Corp. (Calgary, Alberta, Canada). Plasma samples were diluted 1:2 with PBS and the total protein concentration of mammary tissue homogenates was adjusted to 1 mg/mL using radioimmunoprecipitation assay buffer, before being measured.

Statistical Analysis

The concentrations of cytokines in tissue were measured at quarter level and in the plasma at cow level. Goodness of a fit of a normal and lognormal distribution of the data were checked using the Anderson-Darling test, and outliers beyond 3 standard deviations were removed. Cytokine data were log-transformed when not normally distributed and when the transformation improved the A2 score by at least 0.5 before statistical analysis (transformed for data in plasma: IL-1α, IL-6, IL-8, IL-10, IP-10, MCP-1, MIP-1β, TNF-α, and VEGF-A and in tissue: IL-1α, IL-1β, IL-6, IL-8, IL-17A, IL-36RA, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and VEGF-A). Data were back-transformed for visualization. The back-transformed means are the geometric means, and the back-transformed SEM was obtained by multiplying the SEM of the transformed data by the first derivative of the back-transform function, in this case exp(δ) × SEM. Data were analyzed using a mixed model, using time, treatment, and the interaction of time and treatment as the fixed factors and using individual cows and cow pairs (or block) as random factors. Comparisons of the conditions within each time point were performed using pairwise means comparisons using the Dunn-Sidák correction. JMP PRO 15 was used to fit the model, and Prism 9 was used to generate graphs. Significance of the results was declared at adjusted P < 0.05, and a trend was declared when 0.05 < adjusted P < 0.1.

RESULTS

Cytokine Responses to Acute LPS Challenge

The Millipore MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel 1–Immunology Multiplex Assay simultaneously analyzes 15 cytokines (IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, IL-36RA, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and VEGF-A). All 15 cytokines, except IL-4 (sensitivity = 2.56 pg/mg protein; not detectable in 17 of 45 samples), were successfully detected and measured in tissue homogenates at the protein concentration of 1 mg/mL, whereas IL-1β (sensitivity = 2.56 pg/mL, not detectable in 37 of 45 samples), IL-4 (2.56 pg/mL, 43/45), and IL-17A (0.64 pg/mL, 32/45) concentrations were too low to be measured in most plasma samples after 2-fold dilution (Supplemental Table S1; https://doi.org/10.6084/m9.figshare.24886182; Zhao, 2023). The cytokines were not analyzed in mammary tissues of the CU group.

Responses of different cytokines to acute LPS challenge could be characterized as local, being confined to the infused mammary gland, or systemic, affecting the whole body. Plasma concentrations of cytokines considered to show a systemic response differed significantly (P < 0.05) between TRT and CON groups of animals after LPS treatment.
systemic response was evident in the mammary tissue, based on significant \( (P < 0.05) \) differences between TS and CS glands after LPS infusion. Furthermore, significant \( (P < 0.05) \) differences in cytokine concentrations between TL and TS glands or between TL and CS glands after LPS infusion indicated a local response in the infused mammary glands.

**Systemic Cytokine Responses in Plasma.** There was no significant effect of LPS treatment on the plasma concentrations of 8 cytokines, namely IFN-\( \gamma \), IL-1\( \alpha \), IL-8, IL-36RA, IP-10, MIP-1\( \alpha \), TNF-\( \alpha \), and VEGF-A, but the levels of IFN-\( \gamma \), IL-36RA, and IP-10 showed a significant effect \( (P < 0.05) \) of a treatment by time interaction (Figure 1, Supplemental Table S1A). Plasma concentrations of 4 other cytokines, including IL-6, IL-10, MCP-1, and MIP-1\( \beta \), were significantly changed in TRT cows compared with CON cows after LPS treatment, but only MIP-1\( \beta \) showed a significant treatment by time interaction. (Supplemental Table S1A, \( P < 0.05 \)). These 4 cytokines were systemic responders. TNF-\( \alpha \) tended to have a treatment effect \( (P = 0.09) \) and IL-10 tended to have a treatment x time interaction \( (P = 0.07) \). Plasma concentrations of IL-6, IL-10, IP-10, MCP-1, and MIP-1\( \beta \) were elevated dramatically at 3 and 6 h, then declined to the basal level from 12 h to 24 h (Figure 1).

**Systemic Cytokine Responses in the Mammary Gland.** Of all 14 cytokines detected, 8 (IL-13, IL-6, IL-10, IL-17A, IL-36RA, IP-10, MIP-1\( \beta \), and VEGF-A) showed evidence of systemic response in the mammary gland because their overall concentrations were significantly higher in the tissue of TS than CS glands after LPS treatment (Supplemental Table S1B, \( P < 0.05 \)). At 3 h after LPS treatment, the levels of IL-6, IL-10, IL-36RA, MIP-1\( \alpha \), and MIP-1\( \beta \) were higher in TS versus CS glands \( (P < 0.05) \) but those systemic responses were short-lived as there were no significant differences of lymphocytes per alveolus between TS and CS by 12 h (Figure 2). Both IL-17A and VEGF-A remained higher in TS compared with CS at both 3 and 12 h (Figure 2).

**Local Cytokine Responses in the Mammary Gland.** In comparing TL with CS glands, the concentrations of 12 cytokines in the tissue, including IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-8, IL-17A, IL-36RA, IP-10, MCP-1, MIP-1\( \alpha \), MIP-1\( \beta \), TNF-\( \alpha \), and VEGF-A, differed significantly overall after LPS treatment (Supplemental Table S1B, \( P < 0.05 \)), indicating a local response of these cytokines to LPS treatment. Only IFN-\( \gamma \) and IL-10 showed no difference. The concentrations of IL-36RA, MIP-1\( \alpha \), MIP-1\( \beta \), and VEGF-A in TL glands increased dramatically from 3 h to 12 h after LPS infusion (Figure 2). The concentrations of IL-1\( \beta \), IL-6, IL-8, and TNF-\( \alpha \) peaked at 3 h, then dropped but remained higher at 12 h, whereas the concentrations of IL-1\( \alpha \) and IL-17A rapidly increased at 3 h but showed no difference at 12 h in TL glands compared with CS glands. Finally, only IFN-\( \gamma \) showed tissue levels that were lower in TL versus CS glands after LPS treatment at 12 h.

The comparisons of cytokine levels between TL and TS glands indicated that only IL-8, MIP-1\( \alpha \), MIP-1\( \beta \), and TNF-\( \alpha \) showed significant difference after LPS infusion (Supplemental Table S1B, \( P < 0.05 \)).

**Effects of Intramammary LPS Infusion on Mammary Tissue Histomorphology.** Histological analysis is a direct method of evaluation of mammary tissue injury caused by pathogens or chemicals. We analyzed H&E-stained formalin-fixed, lactating mammary tissues obtained before (0 h) and 3 and 12 h after intramammary infusion of LPS or saline. The LPS infusion did not cause inflammation in the TS glands of treatment cows as indicated by the rare presence of neutrophils inside alveolar lumens, but marked pathological changes occurred in TL glands, as shown by the influx of neutrophils into the alveolar and ductal lumens at 3 and 12 h, in comparison to 0 h (Figure 3, Panels A and Cd). In the control cows, the presence of inflammatory cells was rarely seen in either saline-infused or untreated glands (Figure 3, Panels B and C). The presence of blood in the alveolar lumen was evident in some tissue sections obtained at 3 and 12 h, which might result from biopsy because there was no apparent effect of LPS infusion on the accumulation of blood in alveolar lumen of TL glands. The average number of MEC per alveolus did not differ between TL and other glands \( (P = 0.38; \text{Figure 3, panel Ca}) \). Similarly, the area occupied by mammary alveoli (in \( \mu m^2 \)) did not differ among the glands \( (P = 0.26; \text{Figure 3, panel Cb}) \). No changes were observed in the number of lymphocytes per alveolus between any glands \( (P = 0.39; \text{Figure 3, panelCc}) \). The number of neutrophils per alveolus was 5.2- and 7.2-fold higher in TL versus TS glands at 3 and 12 h after LPS infusion, respectively \( (P < 0.01; \text{Figure 3, panel Cd}) \).

**DISCUSSION**

In the present study, we used a powerful cow model to distinguish and investigate the systemic and local responses and potential mediators during acute mastitis. On one hand, we used a mammary unilateral design to study the local effects, which reduced between-cow variation. Each TRT-cow received LPS treatment through intramammary infusion in the ipsilateral side of the front and rear quarters (TL), whereas the contralateral quarters received saline (TS) to serve as a control. The LPS treatment has been widely used to
Treatment cows (LPS, n = 5) were infused with 50 µg of LPS in 10 mL of saline into the front and rear quarters of one udder-half and with saline in the contralateral glands, whereas quarters of control cows (n = 5) received either 10 mL of saline or no infusion. Blood samples were collected at 0, 3, 6, 12, and 24 h relative to infusions. The plasma concentrations of 15 cytokines (interferon gamma [IFN-γ], interleukin 1 alpha [IL-1α], interleukin 1 beta [IL-1β], interleukin 4 [IL-4], interleukin 6 [IL-6], interleukin 8 [IL-8], interleukin 10 [IL-10], interleukin 17A [IL-17A], interleukin 36 receptor antagonist [IL-36RA], interferon gamma-induced protein 10 [IP-10]/C-X-C motif chemokine ligand 10 [CXCL10], monocyte chemoattractant protein 1 [MCP-1]/chemokine [C-C motif] ligand 2 [CCL2], macrophage inflammatory protein alpha [MIP-1α or CCL3], macrophage inflammatory protein beta [MIP-1β or CCL4], tumor necrosis factor alpha [TNF-α], and vascular epithelial growth factor [VEGF-A]) were analyzed using the Millipore MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel 1–Immunology Multiplex Assay. The levels of IL-1β, IL4, and IL-17A were too low to be detected in all or partial samples. Results are reported as mean concentration (pg/mL plasma) ± SEM. The x-axis for all subfigures is “Time (h).” Asterisks indicate significant difference between treatments at individual time points (P < 0.05). *, **, and *** represent P < 0.05, 0.01, and 0.001, respectively.

Figure 1. Dynamic changes in concentrations of cytokines in plasma of lactating cows in response to intramammary infusion of LPS.
Figure 2. Dynamic changes in concentrations of cytokines in mammary tissue homogenates of lactating cows in response to intramammary infusion of LPS. Treatment cows (TRT, n = 5) were infused with 50 µg of LPS in 10 mL of saline into front and rear quarters of one udder-half (TL, n = 5) and with saline into the contralateral glands (TS, n = 5), whereas quarters of control cows received either saline (CS, n = 5) or no treatment. Mammary tissues were collected at 0, 3, and 12 h relative to infusions. Mammary tissue homogenates were analyzed for 15 cytokines (interferon gamma [IFN-γ], interleukin 1 alpha [IL-1α], interleukin 1 beta [IL-1β], interleukin 4 [IL-4], interleukin 6 [IL-6], interleukin 8 [IL-8], interleukin 10 [IL-10], interleukin 17A [IL-17A], interleukin 36 receptor antagonist [IL-36RA], interferon gamma-induced protein 10 [IP-10]/CX-C motif chemokine ligand 10 [CXCL10], monocyte chemoattractant protein 1 [MCP-1]/chemokine [C-C motif] ligand 2 [CCL2], macrophage inflammatory protein alpha [MIP-1α or CCL3], macrophage inflammatory protein beta [MIP-1β or CCL4], tumor necrosis factor alpha [TNF-α], and vascular epithelial growth factor [VEGF-A]) using the Millipore MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel 1—Immunology Multiplex Assay. The levels of IL-4 were too low to be detected in partial samples. Results are reported as mean concentration (pg/mg protein) ± SEM. The x-axis for all subfigures is “Time (h).” Different letters indicate significant difference between treatment means at the individual time points (a–c; \( P < 0.05 \)).
Figure 3. Histological changes of the mammary gland after LPS treatment in lactating dairy cows. Treatment cows (TRT, n = 5) were infused with 50 µg of LPS in 10 mL of saline into front and rear quarters of one udder-half (TL, n = 5) and with saline into the contralateral glands (TS, n = 5; panel A), whereas quarters of control cows (C) received either saline (CS, n = 5) or no infusion (CU, n = 5; panel B), correspondingly. Mammary tissues were collected at 0, 3, and 12 h relative to infusions and analyzed by hematoxylin and eosin staining (panels A–B, 40×). Panel C: the number of MEC per alveolus did not differ in the mammary tissues of TL, TS, CS, and CU glands (a). Mammary alveolar area (b) and number of lymphocytes per alveolus (c) did not vary in the mammary tissues of TL, TS, CS, and CU glands. A spike of neutrophilic infiltration was observed locally in the alveolar lumens of TL glands at 3 h and numbers remained high at 12 h (d). Results in control are reported as mean ± SEM, and different letters (a, b) above individual observations indicate significant difference between treatments at the time point (P < 0.05).
study *Escherichia coli* mastitis in dairy cows (Bruckmaier, 2005; Petzl et al., 2012), and an LPS dosage of 100 µg per cow has been used to induce clinical signs of acute mastitis in lactating dairy cows (Lehtolainen et al., 2003). Comparisons of responses between TL glands and their neighboring TS glands after LPS challenge could show the local effects and factors induced by LPS. However, the local effects and factors resulting from the comparison between TL and TS glands may be affected (masked) by the systemic effects in both glands, as shown in our observations that only 4 of 12 significantly changed cytokines between TL and CS glands were confirmed in comparisons of TL and TS glands. However, we also included a control group in which each cow received saline (CS) and no infusion (CU) in the glands in a fashion corresponding to that of treatment cows. Thus, comparing the tissues of TS and CS glands, as well as comparing the blood of TRT and CON cows, would indicate systemic effects and factors induced by intramammary mammary LPS challenge. We can also compare TL to CS glands for local mammary effects, but this comparison is jeopardized by the systemic effects of LPS in TL glands and possible individual animal variations. Nonetheless, use of both comparisons between TL and TS and between TL and CS can complement each other for studying local effects of LPS in the mammary gland.

In a recent companion study, we reported that LPS induced a significant local response in the injected glands, as demonstrated by dramatic increases in SCC and L-lactate concentration in the milk of TL glands compared with that of TS glands (Shangraw et al., 2020). The local increase in milk SCC is consistent with the large recruitment of neutrophils observed only in the TL glands in this study. However, a systemic effect was also shown by a rise of rectal temperature in TRT cows and similar decreases of milk yield and fat concentration in both TL and TS glands compared with the CS gland (Shangraw et al., 2020). The factors underlying these systemic and local effects are not known although others have suggested cytokine mediators (Shuster et al., 1993; Akhtar et al., 2020) and, in
a second companion study, we reported significant effects on mammary expression of many immune genes (Shangraw et al., 2021). In the present study, we aimed to evaluate the systemic and local cytokine responses to bacterial endotoxin in these cows and to augment our gene expression data with actual cytokine protein concentrations in plasma and mammary tissue. Pathophysiology of acute mastitis is coordinated to balance inflammation and protect against damage to the host. Induction of initial inflammatory reactions is accompanied by production of proinflammatory cytokines and chemokines, but anti-inflammatory signaling pathways also respond. Thus, any responsive cytokines may serve as possible candidates in mediating the observed systemic and local changes.

Despite practicing aseptic technique during infusions, as we did not culture milk from every quarter after infusion of LPS or saline, we cannot exclude the possibility that bacteria were introduced into a gland during infusion. However, it is noteworthy that concentrations of the cytokines measured in this study were stable in plasma and mammary tissue of the control cows which received saline injection. Specifically, none of the 12 cytokines that were measurable in plasma in this study changed significantly over time in CON group cows. Likewise, concentrations of all 14 cytokines analyzed in the tissue homogenates of CS glands were fairly stable over time. These observations indicated that time, intramammary saline injection, and mammary biopsies did not cause significant systemic or local effects on production or accumulation of these cytokines. In addition, the blood seen in some tissue sections at 3 and 12 h of LPS treatment, which apparently resulted from biopsy procedures or tissue inflammation, did not appear to affect the cytokine concentrations in mammary tissue.

Comparison between TL and CS glands showed that acute LPS challenge induced local changes in concentrations of 12 of 14 cytokines detected (IL-1α, IL-1β, IL-6, IL-8, IL-17A, IL-36RA, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and VEGF-A) in the challenged TL glands. The local changes of 4 cytokines (IL-8, MIP-1α, MIP-1β, and TNF-α) were also confirmed from comparison between TL and TS glands. The loss of significant changes in the concentrations of other cytokines between TL and TS glands was likely due to the systemic LPS effect in TS glands. The local changes in IL-6, MCP-1, and TNF-α were also observed in mouse mammary gland after LPS challenge in our recent study (Takashima et al., 2021). Among the affected cytokines, the levels of IL-1α, IL-1β, IL-6, IL-8, MCP-1, and TNF-α increased significantly in TL glands versus TS or CS glands at 3 h after LPS challenge. The responses of these cytokines were transient, their levels decreased or returned to baseline levels at 12 h. These cytokines were early local responders that may promote inflammation in the TL glands. They were likely produced by the mammary cells, not by the recruited leukocytes, because recruited neutrophils remained high in TL glands at 12 h as shown in Figure 3Cd. In addition, IL-36RA, MIP-1α, MIP-1β, and VEGF-A showed a longer response, and their levels became higher in TL glands from 3 h to 12 h. The increased levels of these cytokines could result from LPS-induced local secretion by MEC or recruited neutrophils. Many of these initial inflammatory responses to LPS in bovine mammary gland are consistent with the literature. IL-1α and TNF-α are well known proinflammatory cytokines. IL-1α induces TNF-α signaling and was hypothesized to be the E. coli-specific immune response feature because in vitro stimulation of bovine MEC with S. aureus failed to induce IL-1α expression (Günther et al., 2011). TNF-α may be secreted by the mammary macrophages and by MEC (Riollet et al., 2000). A role for TNF-α in eliciting local and systemic signs of E. coli mediated mastitis has been suggested in lactating cows (Hoeben et al., 2000), and it is associated with the severity of systemic disease during naturally occurring mastitis (Wenz et al., 2010). TNF-α and MCP-1 were induced by LPS in mouse mammary tissue (Yu and Tan, 2019), and MCP-1 mRNA was induced by E. coli treatment in bovine MEC (Zhuan et al., 2020). Moreover, women with clinic and subclinical mastitis have significantly higher levels of MIP-1α and MIP-1β in breastmilk compared with women without mastitis (Farquhar et al., 2005). The increased levels of IL-1α, IL-8, IL-36RA, MIP-1α, and VEGF-A in TL glands but not in the plasma after LPS treatment further indicate that these cytokines are locally produced in response to LPS and play important role in local inflammation. Alternatively, it is possible that these cytokines selectively accumulate in mammary tissue or milk, perhaps being bound or otherwise sequestered in affected glands.

However, the concentrations of IFN-γ tended to be lower in TL glands versus TS glands or CS glands after LPS challenge, consistent with our recent observation in mouse mammary gland (Takashima et al., 2021). IFN-γ is primarily produced by immune cells and known to provide immune response mainly against viruses by preventing their replication within infected cells. Expression of IFN-γ was increased in bovine mammary gland infected with bovine parainfluenza or bovine alphaherpesvirus-1 (Çomakli and Özdemir, 2019), indicating that IFN-γ protects virus-infected mammary glands. Although IFN-γ has long been recognized as a signature proinflammatory cytokine, evidence has also showed the anti-inflammatory aspects of IFN-γ including induction of anti-inflammatory molecules such as...
IL-1 receptor antagonist, IL-18 binding protein, and suppressors of cytokine signaling (Mühl and Pfeilschifter, 2003). Sordillo and Babiuk showed that intramammary infusion of recombinant bovine IFN-γ prevents E. coli growth and inhibits subsequent inflammatory response within the mammary gland in experimental bacterial challenge (Sordillo and Babiuk, 1991). The downregulation of IFN-γ in LPS-challenged glands in this study may promote acute inflammation in these glands. However, the concentrations of IFN-γ were low in both blood and tissue in this study, questioning its changes biologically meaningful.

In addition to those local responses, concentrations of 8 cytokines, including IL-1β, IL-6, IL-10, IL-17A, IL-36RA, IP-10, MIP-1α, and VEGF-A, were increased systemically in TS glands (vs. CS glands) overall (Supplemental Table S1B). The 4 quarters of bovine mammary gland are anatomically isolated, so locally infused LPS can only enter the neighboring glands through systemic blood circulation. Thus, these changes in cytokine concentrations in TS glands must result from LPS or other circulating factors, such as systemically-released cytokines, that diffused into the general circulation. Among these cytokines, IL-10 is well known anti-inflammatory cytokines, and its increase suggests that it may play an anti-inflammatory role to protect against mammary tissue damage in noninfected glands. The functions of systemic increases in the other 6 cytokines in TS glands are not known, but these cytokines may also play protective roles in different functions in noninfected glands. For example, IL-6 may have an anti-inflammatory function via its role as myokine (Pedersen and Febbraio, 2008), and VEGF-A may induce angiogenesis and vasculogenesis (Claesson-Welsh and Welsh, 2013).

Other systemic responses to LPS were evident from changes in the plasma concentrations of 4 cytokines (IL-6, IL-10, MCP-1, and MIP-13). Increases in these 4 cytokines were also observed in mouse plasma after intramammary LPS challenge (Takahshima et al., 2021). Among these cytokines, the increases in concentrations of IL-6, MCP-1, and MIP-13 in blood had a similar trend as their changes in TL glands (vs. CS glands), indicating that the LPS-induced changes of these cytokines in the infused gland may have at least partly contributed to their systemic changes in blood. On the contrary, altered plasma levels of IL-10 were not correlated with tissue concentrations in TL glands, suggesting that this cytokine was mainly released from the cells outside of the infused mammary gland. As noted above, IL-1α, IL-8, IL-36RA, MIP-1α, and VEGF-A concentrations were elevated in homogenates of TL glands (vs. CS) despite LPS having no effect on plasma concentrations. It is possible that cells in LPS-infused glands did not secrete enough of these cytokines to change the systemic concentrations, did not secrete their cargo, or only secreted it after entering into alveolar lumen.

In many cases, cytokine concentrations were significantly higher in TL relative to CS glands, with TS glands often numerically, if not significantly, intermediate to these values, suggesting TL as a local source, with blood as a potential conduit to deliver cytokines to the uninfused glands. This may be at least partly responsible for the depression of milk production in both nonchallenged and challenged glands (Shangraw et al., 2020). Effects of cytokines on milk synthesis by MEC have been reported previously. For example, in response to infection, MEC secrete IL-6 and TNF-α to combat bacterial infection (Lahouassa et al., 2007). TNF-α inhibits lactose synthesis in MEC whereas IL-6 shows both stimulatory and inhibitory effects on milk synthesis (Kobayashi et al., 2016). In a study with a lactating MEC culture model with high lipid production ability, treatment of cells with cytokines such as IL-1β, TNF-α, and IL-6 affected lipid droplet formation and thus the milk synthetic ability of the cells (Matsunaga et al., 2018).

It is also important to point out that only 15 cytokines were analyzed in this study. Many other cytokines may also play important roles in mastitis. For example, we found many other immune genes and pathways that were affected by local, systemic or integrated responses to LPS in these cows (Shangraw et al., 2021).

Finally, our study showed that no histological changes, characterized in terms of MEC per alveolus and alveolar area occupied by mammary parenchyma, were detected within 12 h of LPS infusion. This is consistent with the observation that milk yield was not significantly lower at 12 h but was reduced at 24 h after LPS treatment in our companion study (Shangraw et al., 2020). In addition, it was previously reported that mammary tissue damage does not occur in the first 24 h after bacterial infusion (Brenaut et al., 2014). However, we cannot rule out the possibility that mammary structural changes occur and contribute to the reduction in milk production at later stages. Indeed, we found that LPS increased mammary expression of cleaved caspase 3 and other apoptosis-associated genes (Shangraw et al., 2021). Thus, there might be a delay before such effects on mammary structure are observed or can impair milk yield.

CONCLUSIONS

In this study, we comprehensively profiled 15 cytokines in the blood and mammary tissue along with mammary histological changes in lactating cows in response to local intramammary LPS challenge. Col-
lectively, we showed that the levels of several of these cytokines changed locally in the LPS-challenged glands, whereas concentrations of others were affected in plasma or nonchallenged mammary glands, indicating systemic effects. The latter, presumably, represent candidates for mediating impairment of milk yield and composition in non-LPS-treated glands. However, some of these effects are difficult to rationalize with our results, showing apparent local accumulation of some cytokines in mammary tissue without corresponding changes in plasma concentrations—implying more complexity with respect to the distribution of cytokines between cells, tissues, blood, and milk. Furthermore, LPS infusion elicited heavy migration of leukocytes into the challenged mammary glands only but did not induce detectable changes in mammary tissue histomorphology within 12 h of challenge.

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