SYSTEMIC EFFECTS OF MASTITIS IN THE DAIRY COW: IDENTIFYING MECHANISMS UNDERLYING IMPAIRED LACTATION IN NON-INFLAMED GLANDS

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by
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

SYSTEMIC EFFECTS OF MASTITIS IN THE DAIRY COW:

IDENTIFYING MECHANISMS UNDERLYING IMPAIRED LACTATION

IN NON-INFLAMED GLANDS

presented by Erin Shangraw,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Efficient milk production is temporarily compromised during the inflammatory response to lipopolysaccharide (LPS). Generally, the most severe losses are localized to individual mammary glands with overt cases of mastitis. However, systemic inflammation arising from an inflamed gland can negatively impact lactation in healthy neighboring glands. This interdependence between glands highlights the importance of systemic inflammation in regulating lactation, yet the underlying mechanisms and mediators remain poorly understood. To determine these systemic mechanisms, paired cows were randomly assigned to LPS treatment (T) or saline control (C). Within each treatment, two ipsilateral quarters received one of 2 intramammary sub-treatments: in T cows, infusion of 50μg LPS (T-L) or saline (T-S); in C cows, infusion of saline (C-S) or no infusion (C-N). Front quarters were sampled for milk production and composition while rear quarters were biopsied at 0, 3, and 12 h, relative to infusions. Mammary tissue from 3 and 12 h biopsies was then subjected to RNA sequencing. Results from milk confirmed that LPS induced a characteristic immune response, with a local increase in milk somatic cells and total protein concentrations only in T-L quarters. In comparison, components associated with lactation were affected in both T-L and T-S quarters, with concentrations of milk fat and lactose being lower by 3 and 12 h post-infusion, respectively. Further, milk yields declined steadily over 24 h with similar losses in both T-L and T-S quarters. Induction of transient fever at 3 h, followed by changes in plasma antioxidant capacity and glucose concentrations, provided additional evidence of systemic responses to inflammation. Collectively, these results confirmed that localized

mastitis affected neighboring glands and demonstrated that each milk component was affected by unique time- and treatment- dependent effects.

Analyzing changes in the mammary transcriptome provided further insight into the regulation of the immune response and lactation during mastitis. Most genes were differentially expressed (DE) only in T-L glands and were associated with pro-inflammatory, cell signaling, and metabolic pathways. There was no direct link between expression of lactation-specific genes and hypogalactia or altered milk composition; however, genes unrelated to lactation were upregulated in T-S glands, suggesting that differential expression of other genes may have impacted lactation. At 3 h, the upregulation of negative feedback inhibitors of pro-inflammatory pathways in T-S tissue indicated unidirectional signaling from T-L to T-S glands, likely mediated by cytokines. Later, at 12 h, increased expression in T-S quarters of genes linked to one carbon metabolism and glucocorticoid stimulation indicated that other mediators may also impact milk production. From 3 to 12 h, expression of immune genes diminished in both glands despite increasing somatic cells in T-L quarters. Given the similar decline in quarter milk yields through 24 h, results suggested that an acute, transient inflammatory signal was sufficient to induce delayed effects on lactation. In summary, localized mastitis impaired lactation in non-inflamed, neighboring quarters through both direct and indirect actions of pro-inflammatory mediators.

CHAPTER 1: LITERATURE REVIEW

Introduction

Mastitis remains a prevalent and costly disease affecting the dairy industry. In the United States alone, mastitis is estimated to cost producers over $600 million per year (Hogeveen et al., 2019), of which 35-60% of lost revenue is due to impaired milk production (Aghamohammadi et al., 2018; Hogeveen et al., 2019). Because each quarter in the udder is a separate gland, lower milk yields and poorer quality milk are generally attributed to quarters with active cases of mastitis (Hoeben et al., 2000; Burvenich et al., 2003). However, physical independence does not equal functional independence because milk production can be reduced in all glands by systemic inflammation (Shuster et al., 1991b; Mitterhuemer et al., 2010). Despite decades of research, the mechanisms controlling mammary function during inflammation remain unclear, particularly in clinically healthy glands exposed to systemic inflammation. For the purpose of this review, I will focus primarily on the dairy cow, briefly covering relevant aspects of the physiology of lactation and mammary defense, then addressing the kinetics of local and systemic inflammation on mammary function and the current understanding of factors and genes regulating lactation during mastitis. From there, I will discuss transcriptomic studies as a tool to identify key mechanisms and novel genes, emphasizing the lack of studies in neighboring glands. Ultimately, understanding how systemic inflammation affects mammary function may lead to the development of interventions that enable more efficient milk production without sacrificing the benefits of inflammation.

Overview of Mammary Physiology

The bovine udder is comprised of four anatomically distinct glands. The parenchymal tissue of the left and right udder halves is clearly separated by the median suspensory ligament. The division between fore and rear quarters is less defined but is maintained by a thin connective tissue septum that prevents diffusion of milk between quarters. Further, this division between glands is confirmed by the embryonic development of four separate mammary buds. Consequently, each gland can function individually. However, it is typical for all quarters to respond similarly to an external stimulus, such as temperature, or to an internal stimulus carried in blood. Thus, mammary function is determined by both local and systemic factors (Turner, 1934).

Within a mammary gland, the parenchyma is organized into hollow lobulo-alveolar structures drained by progressively larger ducts that empty into the gland and teat cisterns. Each alveolus is comprised of an inner layer of secretory mammary epithelial cells (**MEC**) and a discontinuous outer layer of contractile myoepithelial cells. Surrounding the alveoli, capillaries deliver nutrients, hormones, and circulating leukocytes, whereas stromal cells provide protection, form structural extracellular matrix (**ECM**), and regulate epithelial cells (Chen et al., 2016). The blood-milk barrier, formed by tight junctions between MEC (Reid and Chandler, 1973), separates milk from extra-alveolar fluid and all stromal tissues except migratory leukocytes.

Milk synthesis and secretion

Total milk production is determined by the number of MEC in the gland and their secretory activity (Capuco et al., 2001). More specifically, the three major milk components, i.e. fat, protein, and lactose, are synthesized by MEC through different

mechanisms, depicted in Figure 1.1. Although milk synthesis and secretion are typically synchronous and maintained within defined ranges, individual components can be regulated independently by unique transcription factors and protein modifications (Osorio et al., 2016). Lower abundance or activity of any enzyme involved in milk synthesis may therefore have a downstream effect on milk production.

Milk fat. Milk fat is the most variable of the major milk components. The lipids present in milk are mainly in the form of milk fat globules (**MFG**) composed almost exclusively of triacylglycerides (**TAG**) surrounded by a tri-layer membrane. In cows, TAG are synthesized by MEC from a pool of *de novo* and preformed fatty acids (**FA**). *De novo* synthesis by the enzymes acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN) contributes short to medium chain FA (C_4-C_{14}). In addition to *de novo* FA, preformed FA are taken up from the blood by FA transporters (Bauman and Davis, 1974; Figure 1.1). The resulting aggregate of FA is transported to the smooth endoplasmic reticulum (ER) for synthesis into TAG through a series of transfers and modifications by lipins and acyltransferases (Osorio et al., 2016). As TAG accumulate, the ER releases them as microlipid droplets contained within a single layer of ER membrane. Lipid droplets coalesce into larger droplets while being transported toward the apical surface. Once associated with the apical surface, lipid droplets begin budding into the lumen surrounded by the plasma membrane (Figure 1.1). Finally, stimulation of myoepithelial cells by oxytocin causes mechanical pinching of the plasma membrane and the release of membrane-bound lipid droplets as MFG (Masedunskas et al., 2017).

Protein. In healthy glands, approximately 80% of the total proteins in milk are caseins. All proteins synthesized by MEC, including caseins, are translated by the rough

ER and transported to the Golgi apparatus to be packaged into vesicles. Synthesis depends on the availability of amino acids and the regulation of mRNA transcription and translation (Rhoads and Grudzien-Nogalska, 2007). After some proteins undergo post-translational modifications, packaged proteins are transported to the apical membrane for release into the lumen through exocytosis (Figure 1.1).

Lactose. The least variable milk component, lactose, is synthesized by the enzyme lactose synthase from glucose and its derivative, UDP-galactose. Two proteins, β1,4-galactosyltransferase 1 (**B4GALT1**) and α-lactalbumin (**α-LA, *LALBA*),** are required to form lactose synthase. Several isoforms of the family of facilitative glucose transporters (GLUT, *SLC2A*), particularly the insulin-independent GLUT1, are expressed in MEC to supply glucose (Zhao, 2014). Lactose synthesis occurs within the lumen of the Golgi apparatus (Kuhn and White, 1975), allowing lactose to be concentrated within vesicles for transport to the apical surface (Figure 1.1). Because lactose cannot diffuse out of vesicle, this concentration of lactose leads to an influx of water, as lactose is the main osmotic molecule controlling water content and milk volume (Stinnakre et al., 1994).

Post-secretion

Between milkings, milk initially accumulates in the lumen of the alveoli, where somatic cells join secreted milk components. In cows, a small but variable proportion of somatic cells are exfoliated MEC, both senescent and viable (Herve et al., 2016). The remainder are leukocytes which migrate through tight junctions between MEC.

Over the course of 12 h, milk is gradually released into the collecting ducts and gland cistern for storage. Notably, this accounts for less than 20% of the total milk held in the udder at milking (Ayadi et al., 2004). Two milk fractions, cisternal and alveolar, can

thus be removed from the gland. Cisternal milk is the first milk to be expressed, requiring only the opening of the teat to drain freely. On the other hand, contraction of the myoepithelial cells in response to oxytocin is necessary to overcome the forces holding alveolar milk in the parenchyma (Bruckmaier and Blum, 1998; Mačuhová et al., 2004). As milk switches from cisternal to alveolar fractions, milk composition changes. Milk lactose and total protein concentrations decline slightly, whereas milk fat concentration increases to nearly 4 times the concentration of pre-milking strip samples (Ontsouka et al., 2003; Nielsen et al., 2005).

Regulation of Lactation

Galactopoiesis, i.e. maintenance of lactation, is controlled by both local factors originating within the gland and systemic factors. A classic example of local control over galactopoiesis is frequency of milk removal, where only glands subjected to the treatment respond. Compared to standard twice-daily milkings, less frequent milking reduces milk yields and secretion rates (Stelwagen and Knight, 1997), whereas additional milkings result in greater milk production (Wall and McFadden, 2007). As such, milking reinforces galactopoiesis for individual glands. In contrast, introducing a substance into a mammary gland through the teat can induce local but anti-galactopoietic effects. Whereas some substances, such as Na^+ or K^+, may affect milk production only in the treated gland (Stelwagen et al., 1999), foreign substances commonly trigger innate immune responses (see Mammary Defense). An immune response may begin as a local response but can rapidly develop into a systemic response, wherein all quarters are affected by systemic factors.

Like local factors, systemic factors may enhance or hinder lactational performance. Galactopoietic hormones, such as prolactin and growth hormone, can maintain or increase milk and milk protein yields. Current evidence suggests that these hormones have direct effects on MEC, increasing their proliferation and inducing the transcription of caseins and α-LA (Lacasse et al., 2016; McCoard et al., 2016). When these galactopoietic signals decline or are opposed by inhibitors, the mammary gland may transition to involution. Involution is characterized by morphological and metabolic changes in the alveoli and MEC. In non-ruminants, involution consists of two phases: a reversible phase where tight junctions between MEC weaken as macrophages arrive to promote an inflammatory environment, and an irreversible phase where MEC commit to apoptosis and the gland is completely remodeled to a non-lactating state (Hughes et al., 2012). In dairy cows, which are typically pregnant during lactation, involution is less drastic; compromised junctions and inflammation develop but apoptosis is moderated by proliferative signals, ensuring a degree of cell turnover and retention that allows involuted quarters to resume milking up to 28 d after milking has ceased (Singh et al., 2015).

Mammary Defense

As an exocrine gland, the mammary gland is exposed to the external environment at every milking. Physical barriers in the teat provide the primary defense against infection. When this defense fails, an array of humoral and cellular defenses stems the multiplication of pathogens and enables their destruction.

Healthy mammary glands are surveilled by a dynamic population of leukocytes. Though the proportion of each cell type fluctuates with stage of lactation and health

status, milk from healthy glands contains a majority of macrophages (20-50%) and lymphocytes (20-60%), followed by polymorphonuclear leukocytes (**PMNL**; 15-30%) (Merle et al., 2007; Schwarz et al., 2011). Macrophages and PMNL are phagocytic cells that coordinate the innate immune response to pathogens and noxious stimuli via cytokines and chemokines. Pleiotropic cytokines promote or inhibit inflammation by activating various intracellular pathways, whereas chemokines, a subset of cytokines, recruit circulating leukocytes to the site of the pathogen or stimulus (Bannerman, 2009). The first leukocytes to be recruited are mainly PMNL, which when activated by pro-inflammatory cytokines release oxidizing agents and cytotoxic enzymes (Burvenich et al., 2003). Once the pathogen is eliminated, macrophages clear damaged and dead cells and release anti-inflammatory cytokines to restore homeostasis (Porcheray et al., 2005; Aitken et al., 2011).

Mammary epithelial cells contribute to mammary defense by coordinating cytokine production with resident leukocytes. Cultured MEC express pathogen recognition receptors and an extensive selection of cytokines when exposed to bacterial challenges (Ibeagha-Awemu et al., 2008; Günther et al., 2011). The immunocompetence of MEC serves a dual purpose: increasing recruitment of leukocytes to the affected gland and providing a mechanism for MEC to respond appropriately to inflammation.

Overview of Mastitis

Mastitis is defined as inflammation of the mammary gland and is predominantly caused by an intramammary infection (**IMI**). Due to the anatomy of the udder, the causative mastitis pathogen is typically isolated only from infected glands. Detection of the pathogen by host recognition of its pathogen associated molecular patterns (**PAMP**s)

triggers the recruitment of PMNL and causes the milk somatic cell count (**SCC**) to rise. In most cases, the mammary gland clears the pathogens with few, if any, symptoms. If, however, the gland is exposed to greater numbers of pathogens or the immune system is compromised, the pathogens can multiply more rapidly and increase the risk of more severe symptoms (Burvenich et al., 2003).

Notably, different mastitis pathogens cause different responses in terms of severity and resolution. An analysis of mastitis incidence on two commercial farms found that Gram-negative coliforms, such as *Escherichia coli* and *Klebsiella* species, provoked greater acute drops in milk yield than more persistent infections caused by Gram-positive bacteria, e.g. *Staphylococcus aureus* (Gröhn et al., 2004). Experimental infections of *E. coli* or *S. aureus* provide greater insight into this difference in severity. For example, one study measuring milk yields before and 24 h after a bacterial challenge found an 84% reduction during *E. coli* infections compared to 30% in *S. aureus* infections (Petzl et al., 2008). This difference can mainly be attributed to the presence of lipopolysaccharide (**LPS**) in *E. coli*, a major structural component of the outer lipid membrane of Gram-negative bacteria. Upon destruction of the bacteria, LPS is released from the outer membrane. Greater amounts of LPS are released from larger colonies of *E. coli* (Van Den Berg et al., 1992), emphasizing that early detection and removal of pathogens is critical to limiting exposure and symptoms. Importantly, LPS itself is not cytotoxic; rather, the host response to the endotoxin determines the downstream effects.

Action of LPS

Each LPS unit contains a hydrophobic lipid A moiety, a core region of oligosaccharides, and a highly variable O-antigen region of repeating polysaccharide

units (Rietschel et al., 1994). The lipid A moiety of bioactive LPS binds to Toll-like receptor 4 (TLR4), an LPS-specific pattern recognition receptor present on leukocytes and epithelial cells (Ibeagha-Awemu et al., 2008). Activation of TLR4 by LPS is catalyzed by three cofactors: LPS-binding protein (LBP), myeloid differentiation-2 (MD2) and cluster differentiation-14 (CD14). Once activated, the signaling cascade induces the activation of nuclear factor κβ (NFKB) transcription factors (Verstrepen et al., 2008).

The highly conserved family of NFKB transcription factors regulate inflammatory and stress-related genes. Inhibitor of κβ (IKB) proteins normally maintain NFKB in an inactive state outside the nucleus. When LPS activates TLR4, Iκβ kinases (IKK) are activated and phosphorylate IKB to release NFKB (Verstrepen et al., 2008). Translocation of NFKB to the nucleus causes upregulation of both pro-inflammatory mediators and regulatory proteins (Bonizzi and Karin, 2004). The resulting collection of pro-inflammatory mediators, acting through different receptors and secondary messengers, reinforce the initial stimulus of LPS binding and activate related inflammatory branches.

Detoxification and Clearance of LPS

Once in the body, the fate of LPS depends on its localization. In blood, LPS can be detoxified through four mechanisms: (i) sequestration by binding proteins, (ii) enzymatic alteration of lipid A, (iii) uptake and catabolism by macrophages, particularly Kupffer cells of the liver, and (iv) modification of cellular responses to a more tolerant state (Hampton and Raetz, 1991; Munford, 2005). In the mammary gland, LPS can be detoxified, cross the blood-milk barrier to enter systemic circulation, or be removed

through milking. Ziv et al. (1976) showed that after administering a high dose of LPS (10 mg into each of two quarters), concentrations of LPS in milk from challenged glands began to fall within 1 h yet remained detectable after 48 h; in comparison, blood remained negative or showed only trace amounts, matching the negligible concentrations in milk from the non-infused glands. A later study found that experimental *E. coli* mastitis induced significant but sporadic increases in plasma LPS in only 3 out of 12 treated cows (Dosogne et al., 2002). This indicates that LPS is either detoxified within the mammary gland, rapidly detoxified upon entering circulation, or both. Therefore, LPS induces local effects but may not have direct systemic effects. Rather, the mediators released in response to LPS may hold the key to unlocking systemic mechanisms.

Altered Physiology During Mastitis

Severe cases of mastitis induced by LPS or *E. coli* infections are characterized by systemic, clinical symptoms. Fever, elevated heart rate, lethargy, reduced feed intake, and hypersalivation can develop within hours of an LPS challenge and persist for several additional hours (Jacobsen et al., 2005; Aditya et al., 2017). Pathophysiological changes in blood accompany physiological responses to LPS, wherein concentrations of cells and molecules may increase (e.g. cytokines, eicosanoids, acute phase proteins), decrease (PMNL), or exhibit variable responses (glucose) (Lohuis et al., 1988; Aitken et al., 2011). Naturally, early studies compared spatiotemporal changes in milk against blood components and clinical symptoms to establish relationships.

Manifestation of Local and Systemic Effects in Milk

The local response of a mammary gland to an intramammary bolus infusion of *E. coli* or LPS is well-documented. A timeline of the earliest identified changes in milk is

presented in Figure 1.2. The earliest changes reflect a change in the permeability of the blood-milk barrier, wherein concentrations of ions and albumin increase in milk, followed closely by migration of immune cells into the lumen (Figure 1.2). Merely 12 h after administration, milk yields of challenged quarters are reduced by 30-75%, with at least 50 times the number of somatic cells as pre-challenge (Hoeben et al., 2000; Mehrzad et al., 2001; Petzl et al., 2008). In comparison, the response of non-inflamed neighboring glands is less drastic, with weak drops in milk yield and slight increases in SCC.

Expanding upon earlier studies reviewed by Lohuis et al. (1988), a series of experiments led by Shuster in Harmon's lab progressed the hypothesis that non-inflamed neighboring glands undergo hypogalactia during LPS-induced mastitis. At 12 h post-infusion, quarter milk yields declined nearly 20% in both LPS-challenged and neighboring saline-infused glands, after which 24 h yields recovered slightly in neighboring glands but continued to fall in LPS-challenged glands to 67% of pre-infusion yields (Shuster et al., 1991c). Further, markers of inflammation (e.g. SCC, albumin, lactoferrin) were increased only in milk from LPS-challenged glands. To account for these changes, the authors proposed that milk production in all glands was affected by "systemic suppression", with an additional "local suppression" related to inflammation in only LPS challenged glands (Shuster et al., 1991c). These researchers recognized, however, that several inflammatory responses occur within the first 12 h of an LPS challenge. Thus, these researchers conducted a second experiment to better characterize the temporal response in milk production to an LPS challenge by increasing milkings from 2 to 4 times daily (Shuster et al., 1991a). Treated and neighboring quarters had a

similar decline in milk production at 12 h post-challenge but not before. Yields of milk components declined as well. Notably, milk fat yields dropped at 6 h, well before changes in other components, suggesting differential regulation of milk components (Shuster et al., 1991a). Having established a more complete timeline, their third experiment focused specifically on the systemic induction of inflammation by recording milk production in response to an intravenous (i.v.) bolus infusion of LPS (Shuster et al., 1991b). Similar to the preceding experiments, milk yields declined by 33% by 12 h post-infusion. Most importantly, there was no indication of an immune response in milk (Shuster et al., 1991b). Therefore, further experiments were warranted to identify the systemic mediators of hypogalactia.

Of the various systemic factors that could play a role in mediating hypogalactia in all quarters, Shuster and colleagues decided to investigate cytokines and glucocorticoids (Shuster and Harmon, 1992; Shuster et al., 1993). Interestingly, both regulate glucose.

In cows, the mammary gland requires plasma-derived glucose for lactose synthesis. Bovine mammary epithelial cells lack the necessary enzyme, glucose-6-phosphatase, to perform intracellular gluconeogenesis (Scott et al., 1976). Given this limitation, lactose yields depend on the amount of glucose supplied to the mammary gland and the utilization of glucose by MEC. Some researchers have proposed that reduced milk yields result from lower glucose availability because cows with mastitis often have hypoglycemia (Lohuis et al., 1988; Waldron et al., 2003). In fact, an activated immune system was estimated to utilize over 1 kg of glucose in the first 12 h following an i.v. infusion of LPS (Kvidera et al., 2017), which should theoretically limit the supply reaching the mammary gland. However, Kvidera et al. (2017) also reported that

maintaining euglycemia during the LPS-challenge did not prevent hypogalactia. Thus, glucose utilization by MEC may be directly regulated by factors present during systemic inflammation whether glucose is limiting or not.

Timing and Mechanisms of Systemic Factors

***Cytokines*.** In blood, pro-inflammatory cytokines are among the earliest indicators of an inflammatory response. Plasma tumor necrosis factor α (TNF-α) increases within 1 h of infusion of LPS (Paape et al., 2002; Waldron et al., 2003), which is earlier than any cytokines have been detected in milk (Figure 1.2). Interleukins (IL) 1 and 6 are elevated within 3-4 h after an LPS challenge (Shuster et al., 1993). In healthy cows, subcutaneous injections of recombinant bovine TNF-α caused a 16% reduction in milk yield, increased IL-1β concentrations, and altered the response of growth hormone, thyroid hormones, and cortisol to their stimulating hormones (Kushibiki et al., 2006). With this association between cytokines and milk production, researchers soon discovered that these cytokines could potentially mediate hypogalactia through control of lactose synthesis, either by dysregulation of glucose transporters or by interfering with mammary utilization of glucose.

Facilitative glucose transporters, particularly insulin-independent GLUT1, are highly expressed during lactation (Komatsu et al., 2005; Zhao 2014). Treatment of cultured murine MEC with TNF-α and IL-1β can induce internalization and degradation of GLUT1 from the basolateral membrane (Kobayashi et al., 2016; Matsunaga et al., 2018), limiting glucose uptake. On the other hand, because mammary glucose transport is mainly facilitative, glucose must be converted into a product, such as lactose, to maintain the concentration gradient into the cell. Conversion of glucose and galactose to lactose

requires both subunits of lactose synthase, B4GALT1 and α-LA. In addition to GLUT1, TNF-α treatment of cultured murine MEC also reduced transcript expression of *LALBA* (Kobayashi et al., 2016). Similarly, bovine mammary tissue after intramammary LPS challenges showed negative regulation of *LALBA* (Schmitz et al., 2004; Gross et al., 2015). This appears to be a direct inhibition of *LALBA* due to inflammation, rather than reduced glucose availability, because altering the concentration of glucose affected expression of *B4GALT1* but not *LALBA* in bovine MEC (Lin et al., 2016). Ultimately, it remains unclear whether lactose production is regulated *in vivo* by cytokines induced by LPS and, if so, whether the mechanism regulates transport and/or utilization of glucose.

Glucocorticoids. In cows administered an i.v. bolus of LPS, plasma cortisol increases in 1-2 h, peaks by 3-4 h, then returns to baseline within 24 h post-challenge (Waldron et al., 2003; Vernay et al., 2012). A similar response in cortisol levels is observed after intramammary administration of LPS (Shuster et al., 1993; Waldron et al., 2006). Notably, treatment of cows with the potent synthetic glucocorticoid, dexamethasone, or supraphysiological doses of adrenocorticotropic hormone (ACTH) decreases milk yield (van der Kolk, 1990; Shamay et al., 2000). As noted for cytokines, Hartmann and Kronfeld (1973) determined that dexamethasone affected milk production because the mammary gland utilized less glucose. It must be noted, however, that physiological doses of ACTH that induce a spike in plasma cortisol comparable to those observed during LPS challenges do not affect milk yield (van der Kolk et al., 1991; Shuster and Harmon, 1992). Thus, if cortisol is involved in the systemic control of milk production, it is likely in conjunction with other factors.

Timing and Mechanisms of Local Response to LPS

Whereas systemic factors should affect all glands, local changes in the inflamed quarter may cause additional losses in milk and milk component yields. Although physical changes in the mammary gland are apparent, the mechanisms behind these effects remain debatable.

Tight junctions*:* During lactation, tight junctions between the lateral membranes of MEC limit paracellular transit of blood and milk components. Greater recovery of albumin and Na^+ in milk (Lengemann and Pitzrick, 1986), in conjunction with elevated concentrations of α-lactalbumin and lactose in plasma and urine (Shuster et al., 1991c; Wellnitz et al., 2015), is therefore associated with an increase in permeability, or leakiness, of the blood-milk barrier. Perhaps the simplest explanation for hypogalactia in glands with leaky tight junctions is the loss of lactose into blood. As the major osmolyte in milk, a smaller amount of lactose present in milk would theoretically reduce the volume of milk. However, blocking the leakiness of tight junctions and escape of lactose into blood with ACTH did not lessen the reduction in milk yield associated with milk stasis (Stelwagen et al., 1998). Indeed, an imbalance in the Na:K ratio in milk also impairs milk production. Increasing Na^+ concentrations in healthy quarters reduced milk lactose concentrations and milk yield without affecting tight junction integrity or plasma lactose (Stelwagen et al., 1999). Therefore, it is unlikely that the loss of lactose through leaky tight junctions is a major factor causing hypogalactia.

Somatic Cells*:* Milk SCC is significantly increased within 3 h of an LPS challenge (Wellnitz et al., 2015). Furthermore, the distribution of somatic cells shifts from a heterogeneous mixture of macrophages, lymphocytes, and PMN to a majority of

PMN within 6 h post-challenge (Mehrzad et al., 2001; Schwarz et al., 2011). This drastic change in somatic cell composition and concentration is associated with compositional (Fernandes et al., 2004) and metabolic (Xi et al., 2017) changes in milk from inflamed glands. One factor in this response is the increase in enzymes produced by activated PMNL. Plasmin concentration and proteolytic activity in milk closely follows the rise in SCC after intramammary administration of LPS (Mehrzad et al., 2005). Collectively, these enzymes act upon a broad range of substrates, from caseins to components of connective tissue. Activation of the plasmin system and hydrolysis of caseins has been proposed to explain the local suppression of milk synthesis because hydrolyzed casein significantly increased Na^+ and reduced lactose concentrations by 8 h after infusion (Shamay et al., 2003). On the other hand, degradation of the extracellular matrix anchoring MEC triggers apoptosis, impairing overall milk production through reduced synthetic capacity of the gland.

Another factor connected to elevated somatic cells is the release of reactive oxygen and nitrogen species (RONS) by PMNL. Production of RONS by milk somatic cells can increase significantly after intramammary administration of LPS, even compared to production by circulating leukocytes (Mehrzad et al., 2001). The local, indiscriminate release of RONS by PMNL results in oxidative damage to all biomolecules, whether of bacterial or host origin. Thus, RONS that destroy pathogens may also damage MEC and impair milk production. For example, administration of RONS to cultured human mammary alveolar structures induced apoptosis of the luminal MEC but not basal layers (Thomas et al., 2011). It must be noted, though, that this mechanism might not translate across species into an *in vivo* loss of MEC in cows.

Lastly, the release of RONS into surrounding tissues and circulation during mastitis can induce oxidative stress, wherein antioxidants are inadequate to counter oxidants (Ibrahim et al., 2016). However, it remains unknown whether oxidative stress directly regulates mammary function or is merely a consequence of inflammatory mediators (Sordillo and Aitken, 2009).

In searching for the local and systemic factors that regulate milk production during mastitis, results are equivocal. The most promising mediators of hypogalactia in neighboring quarters are pro-inflammatory cytokines, yet the underlying mechanisms regulating lactose synthesis remain unclear. Additionally, the most frequently studied factors largely ignore changes in other milk components, particularly milk fat. Studies must continue to investigate the effects of these and novel factors on lactating mammary tissue to understand the regulation of lactation in response to systemic inflammation.

Gene Expression and Transcriptomics to Study Impact of Mastitis on Lactation

Due to the diversity of potential factors involved in the local and systemic regulation of mammary physiology, research has recently turned to broad approaches to identify targets of interest and mechanistic relationships. Microarrays and RNA sequencing are powerful tools to assess global gene expression and answer complex biological questions. Unsurprisingly, the main focus of transcriptomic studies on mastitis has been to unravel the immune response, as reviewed by Rinaldi et al. (2010b). Most studies have emphasized the kinetics of the immune response to different mediators, such as LPS, *E. coli,* or *S. aureus,* in order to better understand the host response (Table 1.1). With few exceptions, glands with acute mastitis show an increased expression of immune responsive and pro-inflammatory genes. From a phenotypic standpoint, this matches the

local response observed in milk. What differs between different mediators are the kinetics of the immune response and the specific genes that are altered. For example, studies that infused mammary glands with LPS identified differential expression of genes for cytokines, chemokines, and acute phase proteins hours earlier than studies that infused glands with *E. coli* (Zheng et al., 2006; Mitterhuemer et al., 2010; Buitenhuis et al., 2011). Fewer genes were differentially expressed in response to *S. aureus* than to *E. coli* in primary bovine MEC (Günther et al., 2011), matching the severity of responses in milk yield. Moreover, even within an inflamed gland, different regions of the mammary gland responded differently over time, with expression of pro-inflammatory genes appearing first in teat tissues and later in distal parenchyma (Rinaldi et al., 2010a; Petzl et al., 2016). Thus, the local transcriptional response to a pathogen can be distinct but at the same time induce common inflammatory pathways.

Several studies looked beyond the local responses within the inflamed mammary gland to investigate systemic effects, including changes in the liver during experimental mastitis (Jiang et al., 2008; Minuti et al., 2015; Moyes et al., 2016). Similar to mammary tissue, inflammatory and immune responses were the earliest pathways affected in the liver. Further, the expression patterns of genes encoding acute phase proteins and metabolic enzymes confirmed previous evidence of elevated concentrations of acute phase proteins and altered hepatic metabolism during mastitis (Lohuis et al., 1988; Waldron et al., 2003).

For the liver to respond to a distant, localized source of inflammation, it is logical to assume that other organs may respond as well. In reviewing the literature, however, few transcriptomic studies on mastitis have been designed to assess systemic changes in

the non-inflamed neighboring glands, either by comparison against a pre-challenge sample or to a separate, healthy animal. Out of 9 *in vivo* transcriptomic studies, only one by Mitterhuemer et al. (2010) utilized an appropriate design to quantify changes in neighboring glands and none compared responses against a pre-challenge sample from the same animal (Table 1.1). To assess a local inflammatory response, it is valid to compare a challenged quarter against an unchallenged, healthy quarter from the same animal; the popularity of this comparison is reflected in the typical design for these studies (Table 1.1). The issue with this comparison arises when attempting to determine underlying mechanisms of systemic responses, including hypogalactia, without an appropriate control for neighboring samples.

Recognizing the need to understand the kinetics of LPS effects on gene expression, Schmitz et al. (2004) employed a biopsy technique for frequent sampling of mammary tissue. One rear quarter was infused with 100μg LPS whereas the contralateral gland was infused with saline. Biopsies were collected every 3 h, starting just prior to infusions, to measure expression against 0 h in both treated and neighboring control quarters. Using qPCR, the authors confirmed a local immune response in LPS-challenged glands by the upregulation of inflammatory mediators (*TNF-α* and *COX2*) at 3 h post-challenge. Further, genes specific to lactation (*LALBA* and *CSN3*) were significantly downregulated by 9 h in the challenged glands. Interestingly, when the authors analyzed the adjacent control quarters, minor but similar changes in *TNF-α* and *LALBA* expression were apparent, indicating some systemic effects of the immune response on lactation (Schmitz et al., 2004). However, a limitation of this study was the lack of a synchronous negative control, which meant that all changes in gene expression over time were

potentially confounded with biopsy- or saline-induced inflammation. Additionally, the response in milk from sampled quarters could not be measured over time due to blood contamination caused by biopsies.

A later design by Mitterhuemer et al. (2010) used a different approach to determine the systemic effect of *E. coli* mastitis on neighboring glands. In two groups of healthy cows, a single quarter was infused with *E. coli*. After 6 or 24 h, cows were sacrificed to collect mammary tissue from the *E. coli*-challenged and neighboring glands. A third group of cows was maintained under the same conditions but received infusions of saline to allow collection of healthy control tissue. By comparing tissue from cows with mastitis against healthy tissue, the authors not only identified the expected local inflammatory response to an *E. coli* infection but also 476 differentially expressed genes in the neighboring glands, of which 294 were identified in both quarters. Crucially, these common, systemically regulated genes were associated with a protective immune response and with the negative regulation of lactation (Mitterhuemer et al., 2010). Moreover, the unique experimental design allowed milk to be collected prior to tissue harvest, unlike in the design used by Schmitz et al. (2004), allowing actual decreases in milk yields from both challenged and neighboring glands to support changes in gene expression.

A third study by Jensen et al. (2013) supported the influence of systemic inflammation on non-inflamed, neighboring quarters after failing to find an expected local immune response. In this experiment, cows were administered three intramammary infusions, one each in the right front, right hind, and left hind quarters, at three timepoints over 24 h, leaving the final quarter as a non-infected control. Cows received either *E. coli*

or *S. aureus* infusions for comparison of the inflammatory response to each pathogen. However, the small number of differentially expressed genes in challenged quarters compared to the within-animal control, especially for *S. aureus* infections, led the authors to run *post-hoc* analyses on the different control quarters. In doing so, they found 187 differentially expressed genes in "control" quarters, suggesting that gene expression was not stable but differed depending on the type of infection in challenged glands (Jensen et al., 2013). Further, over 25% of the genes identified the *E. coli* control glands were also systemically regulated in the study by Mitterhuemer et al. (2010), indicating that responses to systemic inflammation in neighboring glands can be unique to different infections.

The slow transition in transcriptomic studies from an exclusive focus on local inflammation to more subtle systemic responses is a natural progression as more powerful techniques become available and new studies build upon previous findings. Having reliable annotations to compare against allows researchers to validate new models and pursue novel findings. Indeed, recent studies by Brenaut et al. (2012, 2014) suggested that isolation of RNA from milk fat provides a non-invasive method for frequently sampling the MEC transcriptome. Compared against RNA from whole mammary tissue, RNA from milk fat had lower contamination from immune cells but a similar expression of genes required for milk synthesis (Brenaut et al., 2012). In response to *S. aureus* infection, immune-responsive genes were mainly up-regulated (Brenaut et al., 2014), indicating a direct immune response by MEC previously found in cultured MEC (Günther et al., 2011). On top of this transcriptional validation, the non-invasive aspect is intriguing both as a method to improve understanding of kinetic and spatial

changes to MEC *in vivo* and to simultaneously collect milk production data for biological validation.

Overall, each transcriptomic study has brought a different aspect to the regulation of the mammary gland. As the majority have advanced our understanding of the local mammary response to inflammation, so it is important to continue such investigations into the response of neighboring glands to systemic inflammation. If subtle changes can be found in glands neighboring inflamed glands, perhaps other systemic sources of inflammation affect gene expression as well.

Summary

Our understanding of the regulation of lactation during mastitis has progressed over the past century with advancements in technology, from clinical observations to biochemical analyses to transcriptomics. Each study has built upon previous findings, yet the factor or factors causing transient hypogalactia in both inflamed and neighboring quarters remain elusive. Observational and hypothesis-driven studies provided the first evidence that neighboring glands could also be affected during a localized case of mastitis. As connections between potential mediators and responses grew, targeted studies of cytokines and glucocorticoids provided incomplete clues to explain how hypogalactia occurs during mastitis. Most recently, a few global transcriptomic studies have highlighted the changes that occur in neighboring quarters, but the development of this transcriptomic response to LPS-mediated inflammation has yet to be measured over time. Thus, this experiment was designed to compare spatiotemporal changes in milk production against potential systemic factors and transcriptomic data to identify the systemic mediators affecting lactation. The first objective was to determine the local and

systemic responses of lactating mammary glands to an LPS challenge over the initial 24 h following the challenge. Within this context, the second objective was to determine how the mammary transcriptome of neighboring glands responds to systemic inflammation over time.

Table 1.1. Comparison of global transcriptomic studies on mammary tissue after experimental mastitis challenge

Source	Species	Type	Tissue[1]	Timing[2], h	Control Quarter[3]		Other tissue
					Internal	External	
Zheng et al., 2006	Mouse	LPS	MAP	4	Y	N	N
Minuti et al., 2015	Cow	LPS	Biopsy	2.5	N	Y	Liver
Mitterhuemer et al., 2010	Cow	*E. coli*	MAP	6, 24	Y	Y	N
Rinaldi et al., 2010a	Cow	*E. coli*	MAP/teat	12, 24	Y	N	N
Jensen et al., 2013	Cow	*E. coli/S. aureus*	MAP	6, 12, 24, 72	Y	N	N
Moyes et al., 2016	Cow	*E. coli*	Biopsy	24	Y	N	Liver
Buitenhuis et al., 2011	Cow	*E. coli*	Biopsy	24, 192	Y	N	N
Brenaut et al., 2012	Goat	*S. aureus*	MFG	6, 12, 18, 24, 30	Y	N	N
Brenaut et al., 2014	Goat	*S. aureus*	MFG	6, 12, 18, 24, 30	Y	N	N
Günther et al., 2011	Cow	*E. coli/S. aureus*	pbMEC	1, 3, 6, 24	-	-	-

[1]Mammary alveolar parenchyma (MAP), milk fat globules (MFG), primary bovine mammary epithelial cells (pbMEC)

[2]Time of mammary tissue sampling after intramammary challenge

[3]Control quarter represents samples collected from unchallenged quarters in the challenged animal (internal) or from a healthy animal (external)

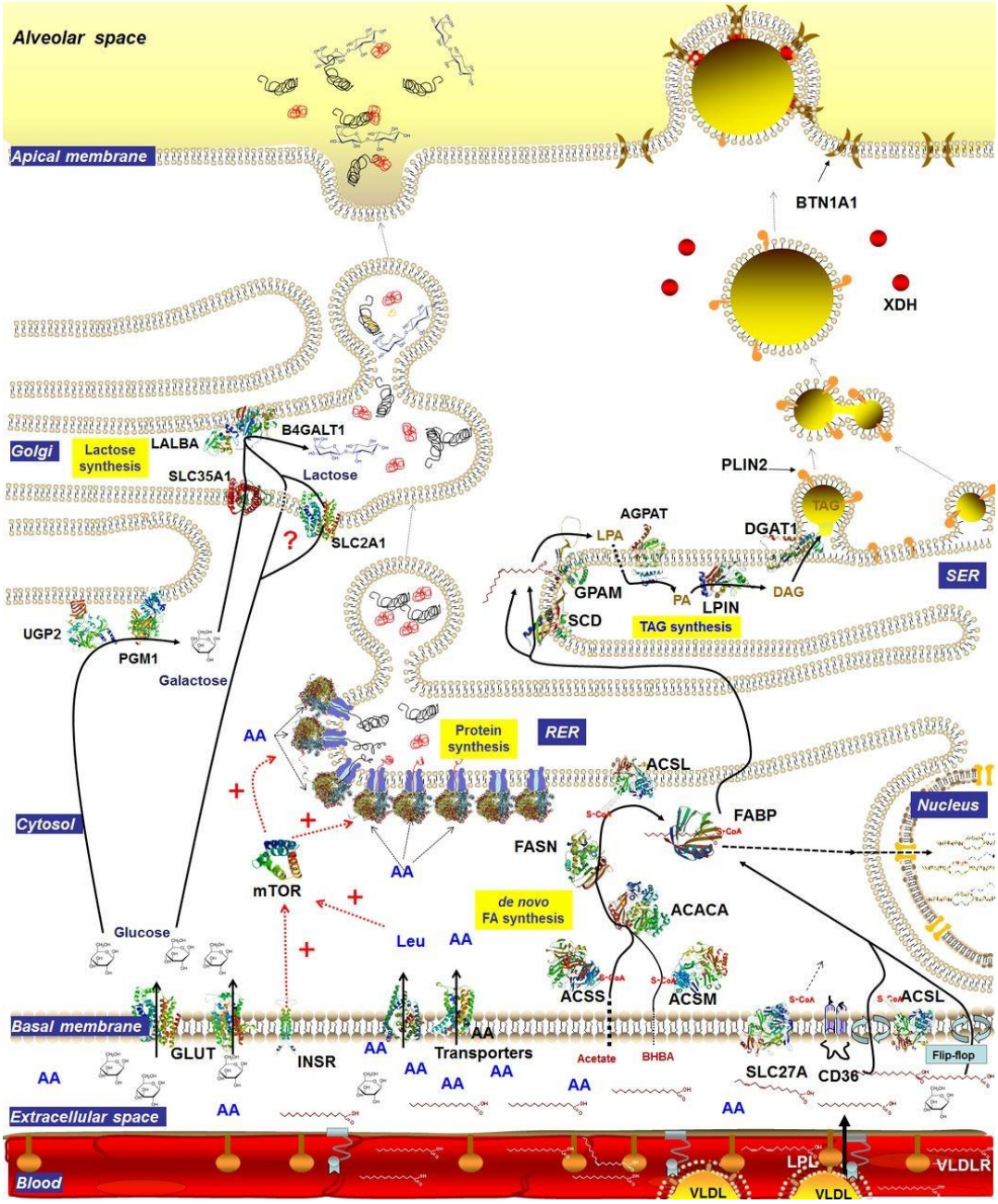

Figure 1.1. Milk fat, protein, and lactose synthesis in the mammary epithelial cell. Milk fat synthesis: All fatty acids (FA) are either formed *de novo* from acetic acid and β-hydroxybutyrate (BHBA) in the cytosol or taken up preformed from blood. Triacylglycerol (TAG) synthesis in the smooth endoplasmic reticulum (SER) requires a series of acyl transfers and modifications. Once formed, TAG coalesce to form microlipid droplets. Lipid droplets released from the SER interact with other lipid droplets and the apical membrane prior to budding into the lumen. Acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN), lipoprotein lipase (LPL), very-low density lipoproteins (VLDL), long-chain fatty acids (LCFA), fatty acid transporter (SLC27A), fatty acid binding protein (FABP), stearoyl-CoA desaturase (SCD), glycerol-3-phosphate

acyltransferase (GPAM), 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), lipin (LPIN), 1,2-diacyl-sn-glycerol (DAG), diacylglycerol O-acyltransferase (DGAT1), adipophilin (or perilipin 2; PLIN2), xanthine dehydrogenase (XDH), butyrophilin (BTN1A1), acyl-CoA synthetase (ACS), fatty acid translocase (CD36);. Protein synthesis: Amino acids (AA) are imported into the cytosol (Shennan and Boyd, 2014). Activation of ribosomes for translation of milk proteins is regulated by the mammalian target of rapamycin (mTOR) and some AA, such as leucine (Leu). Newly formed proteins are shuttled to the Golgi apparatus by vesicular transport for further posttranslational modifications. The mature proteins are packed within exocytotic vesicles and transported to the apical membrane, where they are released into milk. Lactose synthesis: Glucose transporters (GLUT or SLC2A) facilitate uptake of glucose from the basolateral membrane. Synthesis occurs in the Golgi, where the interaction of β1,4-galactosyltransferase 1(B4GALT1) and α-lactalbumin (LALBA) forms lactose synthase, which synthesizes lactose from glucose and UDP-galactose. Glucose can be directly transported into the Golgi or converted first to UDP-galactose in the cytoplasm, then transported by UDP-galactose transporter 2 (SLC35A2). Uridine-diphosphate (UDP); UDP-glucose pyrophosphorylase 2 (UGP2); and phosphoglucomutase 1 (PGM1). Figure from Osorio et al. (2016).

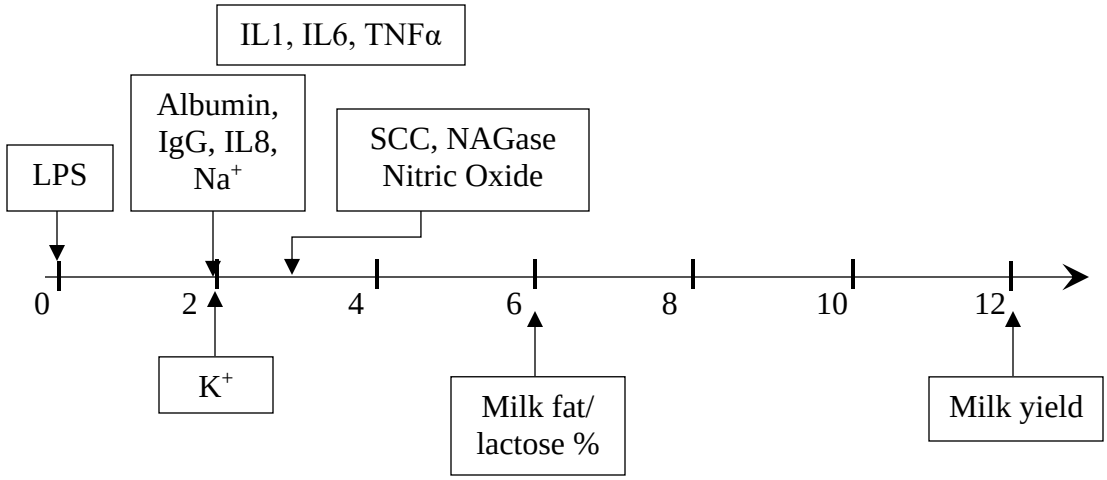

Figure 1.2. Timeline of earliest known changes in milk following intramammary infusion of LPS. Following intramammary infusion of LPS, the earliest evidence of mastitis in milk begins with increases in concentrations of blood-borne proteins, including albumin and immunoglobulin G (IgG), and Na^+ (Lengemann and Pitzrick, 1986; Shuster et al., 1993; Wellnitz et al., 2015). The first detection of cytokines varies between 2 to 4 h depending on the dose of LPS, the specific cytokine, and sensitivity of the assay (Shuster et al., 1993; Persson Waller et al., 2003). By 3 h, milk SCC is elevated compared to control quarters and continues to rise, peaking 12-24 h post-challenge (Mehrzad et al., 2001; Wellnitz et al., 2015). The increase in β-N-acetyl-glucosaminidase (NAGase), a hydrolytic enzyme, correlates with SCC (Bouchard et al., 1999). This is then followed by decreased concentrations of milk fat and lactose. Finally, the effects of LPS culminate in reduced milk yields at 12 h, which may decline further (Shuster et al., 1991a).

CHAPTER 2: INTRAMAMMARY LIPOPOLYSACCHARIDE INFUSION INDUCES LOCAL AND SYSTEMIC EFFECTS ON MILK COMPONENTS IN LACTATING BOVINE MAMMARY GLANDS

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Abstract

Each quarter of the bovine mammary gland is an anatomically and functionally distinct gland. However, mastitis in one quarter affects the function of adjacent, uninfected glands. To investigate the mechanisms and potential mediators of these effects, we quantified early responses of the mammary gland to intramammary LPS challenge, distinguishing between local and systemic effects. Ten multiparous cows over 70 days in milk were blocked into pairs by breed, cow-level somatic cell count (SCC), and milk yield. Within block, one cow was assigned to LPS treatment (T), such that both the front and the rear quarter of a randomly selected udder-half received an infusion of 50 μg LPS in 10 mL saline, these quarters were designated (T-L); the contralateral quarters received only 10 mL saline and were designated (T-S). Similarly, each paired control cow (C) received either 10 mL saline (C-S) or no infusion (C-N), into udder halves. Cows were quarter-milked twice daily, with foremilk samples (~30 mL, front quarters) taken at -24, 0, 3, 6, 12, and 24 h relative to infusions. At 24 h, average milk yield in T-L and T-S quarters fell to 23 and 32% of pre-infusion levels, respectively. For T cows, systemic effects were observed by 3 h post-infusion as rectal temperature was elevated and foremilk fat concentration was reduced in both T-L and T-S. However, SCC and concentrations of L-lactate and total protein in foremilk indicated a local response to LPS: protein was transiently higher at 3 h while SCC and lactate were higher at 6 h in T-L compared to T-S. Lactose concentration showed a local effect at 6 h, being lower in T-L than in T-S, and then a systemic effect at 12 h, being lower in both T-L and T-S than C quarters. Concomitant with changes in milk, systemic effects were also observed in blood. Plasma antioxidant potential and glucose concentration were lower in T than C

cows at 6 or 12 h, respectively, although neither variable remained different at 24 h. In summary, unilateral LPS infusion induced distinct, time-dependent effects on each milk component. Depending on the component, effects were local, systemic, or both, suggesting involvement of multiple, different mediators that collectively result in systemic inhibition of milk production.

Introduction

In dairy cows, acute coliform mastitis remains an obstacle to efficient milk production, premium milk quality, and animal health. Infection by coliforms, particularly *Escherichia coli*, often leads to the rapid onset of clinical symptoms and altered production in infected quarters, comprising hypogalactia, elevated SCC, and reduced milk components (Burvenich et al., 2003). Mastitis places a financial and managerial burden on producers, who must absorb the costs of lost milk production while making swift and appropriate decisions to prevent further losses and restore cow health. To aid decision-making, research involving experimentally-induced mastitis has greatly expanded knowledge of the disease, whether through infection by live bacteria (Hoeben et al., 2000; Mitterhuemer et al., 2010) or infusion of bacterial-derived, pro-inflammatory lipopolysaccharides (**LPS**; Guidry et al., 1983; Shuster et al., 1991b). Similar to severe cases of mastitis, LPS infusion affects not only the infused glands but also the uninfused adjacent glands, termed the systemic effect (Shuster et al., 1991a; Hoeben et al., 2000). However, due to the complex pathophysiology of mastitis, the cause of these systemic responses remains incompletely understood.

Each quarter of a bovine udder is an anatomically independent, functionally distinct gland. Generally, after unilateral experimental infusion, bacteria can only be

isolated from the milk of infused quarters while cultures from neighboring quarters remain negative (Mitterhuemer et al., 2010), illustrating that milk and bacteria are unable to cross from one gland into another. For this reason, many studies have used individual quarters or udder-halves as separate experimental units to compare treatments within cow (Bouchard et al., 1999; Persson Waller et al., 2003; Schmitz et al., 2004). However, when one gland is infused with LPS, the endotoxin itself, along with several pro-inflammatory molecules, can escape into blood (Ziv et al., 1976) and lymph (Persson Waller et al., 2003). Supporting this evidence of systemic inflammatory mediators, several studies have argued against quarter independence, noting increased SCC (Schultze and Bramley, 1982), altered milk composition (Paixão et al., 2017) and differentially expressed genes (Mitterhuemer et al., 2010) in adjacent glands, which were previously presumed unaffected.

Several milk components, including fat, protein, and lactose, are known to change depending on quarter health status (Bruckmaier et al., 2004; Nielsen et al., 2005) or in response to LPS. Additionally, most responses to an LPS challenge occur within the first 24 h (Guidry et al., 1983; Shuster et al., 1991a). However, despite the rapid onset of clinical symptoms, few studies have included measurement of milk components at intervals earlier than 12 h post-challenge. Experiments that included sampling earlier and more frequently than 12 h have been focused mainly on immune responses (Shuster et al., 1993; Bouchard et al., 1999; Persson Waller et al., 2003), although two included measurement of proteins (Wellnitz et al., 2015) and lactose (Guidry et al., 1983). To our knowledge, 6 h post-LPS infusion is the earliest timepoint at which milk composition has been examined and results indicated that different milk components are regulated

independently at this time (Shuster et al., 1991a). Thus, acute changes in milk composition provoked by an LPS challenge are worth investigating to understand local and systemic effects.

Our long-term goal is to identify mechanisms causing the acute local and systemic reactions of mammary glands to intramammary LPS. In the present experiment, we designed a unilateral challenge model with sample collection at 3 and 6 h post-infusion to capture relatively early temporal responses. Our objectives were to determine if and when each milk component responded to local or systemic effects over an acute time course so that relationships between components and potential mediators could be identified. Further, we tested several previous hypotheses proposed to explain the systemic changes in lactation.

Materials and Methods

All procedures involving animals were approved by the University of Missouri Institutional Animal Care and Use Committee (Protocol #9283).

Animals and Management

Ten multiparous Holstein-Friesian (n = 8) or Jersey x Holstein (n = 2) cows were paired by breed, udder-level SCC, and daily milk yield. All cows included in the study had cow-level SCC < 174,000 cells/mL based on the most recent monthly DHIA testing (Mid-South Dairy Records, Springfield, MO) and all had reached mid-lactation (range: 157 to 373 DIM) except one (75 DIM). All quarters of eligible cows were aseptically sampled at d -14 and -8 prior to infusions and all quarters of enrolled cows were culture-negative for major mastitis pathogens.

Cows were moved from free-stall housing and temporarily housed in a straw-bedded pack barn. Cows had free access to water and were fed ad libitum a TMR formulated to meet or exceed lactational requirements. All cows were milked twice daily prior to and during experiment. For two mornings prior to initiation of the experiment, cows were milked in a portable chute to familiarize each cow with the novel routine.

Design

Paired cows were randomly assigned to one of two cow-level treatments: 1) LPS treatment (**T**) or 2) control (**C**). Within the respective treatment, udder-halves were assigned one of two sub-treatments (Figure 2.1). For T cows, the left or right udder-half was randomly assigned to receive an infusion of 50 µg *E. coli*-LPS (serotype O55:B5; Sigma-Aldrich, St. Louis, MO) in 10 mL 0.9% sterile saline into both of the ipsilateral front and rear quarters; these quarters were designated (**T-L**). The contralateral quarters received only 10 mL sterile saline and were designated (**T-S**). For C cows, ipsilateral quarters of one udder-half received infusions of 10 mL saline and were designated (**C-S**), whereas the contralateral quarters were not infused, and designated (**C-N**). All quarters of each cow were infused within 1 min, defined as 0 h.

Cows were quartermilked at 12 h intervals, relative to infusions. Individual quarter milk samples were collected from front quarters at milkings and at 3 and 6 h post-infusions. Rectal temperature and blood samples were collected at milk sampling times. As part of another study, cows were sedated and both rear quarters were biopsied at 0, 3 and 12 h post-infusions. Biopsies compromised the blood-milk barrier thus precluding meaningful measurement of milk components in blood as indicators of inflammation.

Accordingly, milk yield and composition was determined from the non-biopsied, front quarters.

At the morning milking on the initial day of the experiment, cows were randomly assigned a milking order. After milking, cows were restrained in a stationary stanchion and sedated by i.v. injection of 20-30 µg/kg BW of xylazine (Akorn Inc., Lake Forest, IL) in preparation to obtain mammary biopsies from both rear quarters, following the method of Farr et al. (1996). Immediately after biopsy, quarters were aseptically infused with LPS or saline or remained uninfused according to the experimental design described above. All cows were infused approximately 49 min after milking (range: 30 to 90 min). After infusion, teat ends were held closed and the udder was massaged for 10 s to distribute solutions. Cows were released back to the pack barn with free access to TMR and water until the next sampling time.

Milking and Milk Sampling

For milk sampling (-24 and -1 h pre-infusion; 3, 6, 12, and 24 h post-infusion), teats were dipped in iodine, manually stripped 2-3 times, and thoroughly dried with a clean paper towel. Then, 25-30 mL foremilk was manually expressed into a container and preserved with a bronopol tablet for DHI analysis. Immediately thereafter, an additional 80-90 mL was collected into two 50 mL conical tubes. When sampling coincided with milking times (-24 and -1 h pre-infusion; 12 and 24 h post-infusion), the quarter milking unit was attached immediately after foremilk collection and the remaining milk was harvested by portable machine milking into collapsible bottles corresponding to each quarter. Cows were milked out with 10-20 IU of oxytocin given i.v. after unit attachment. After milking, each milk bottle was weighed to obtain milk yields per quarter, then

agitated before removal of 25 mL milk for DHI analysis. Thus, two types of quarter milk samples were collected: 1) foremilk, representing strippings taken before machine milking and 2) harvested milk, defined as samples drawn from bottles after milking.

Preserved milk samples were left at ambient temperature up to one week until shipment for milk composition analysis (Mid-South Dairy Records, Springfield, MO). Milk from one 50 mL tube was centrifuged at 2,200 *g* for 15 min at 4°C within 5 min of collection to separate milk fat, then 1 mL aliquots of skimmed milk were frozen at -20°C, and stored at -80°C.

Blood Sampling

At 1 h pre-infusion and 3, 6, 12, and 24 h post-infusion, blood was taken from the coccygeal vein into vacutainer tubes (Covidien, Mansfield, MA) for serum (no additive) and plasma (K_3-EDTA). Plasma was processed within 5 min of collection whereas serum was refrigerated overnight at 4°C. Samples were centrifuged at 2,200 *g* for 15 min at 4°C. Supernatants were aliquoted into 1.5 mL tubes and stored at -80°C.

Assays

Milk L-lactate was measured in skimmed milk as described by Shapiro and Silanikove (2010) using L-lactic dehydrogenase (Sigma, St. Louis, MO) and diaphorase (Worthington Biochemical Corp., Lakewood, NJ). Fluorescence was read at Ex/Em = 530/590. Intra- and inter-assay coefficients of variation were 4.8 and 5.4%.

Urea nitrogen concentration was analyzed in serum and skimmed foremilk samples using the colorimetric BUN procedure no. 580 (Stanbio Laboratory, Boerne, TX), which is based on acid catalyzed diacetyl monoxime methodology. Serum was analyzed directly. Milk was diluted 1:5 with the supplied acid reagent

(sulphuric/phosphoric acids), then deproteinized by centrifugation at 17,000 x *g* for 5 min. The clear milk/acid supernatant was then analyzed. Absorbance was read at 520nm. Intra- and inter-assay coefficients of variation were 1.8 and 2.3% for serum, and 3.2 and 1.7% for milk, respectively. For validation, selected milk samples were spiked with 3 known concentrations of urea nitrogen and subjected to precipitation and analysis. Spike recoveries averaged 113 ± 19% over a range of 11.5 to 26.2 mg/dL.

The antioxidative potential of blood was analyzed by the ferric reducing ability of plasma (FRAP) assay adapted from Benzie and Strain (1996). Working FRAP reagent was freshly prepared and plasma was diluted 1:2 with double distilled water. Forty µL of the diluted plasma or standard was incubated in 200 µL FRAP reagent for 20 min before reading absorbance at 593nm. Known aqueous concentrations of Fe^{2+} (50-500 µM) were used to generate a standard curve. Intra- and inter-assay coefficients of variation were 4.2 and 2.4%.

Plasma glucose was analyzed using PGO enzymes and o-dianisidine dihydrochloride, according to manufacturer's instructions (Sigma, St. Louis, MO). Five µl of sample or standard was incubated in 200 µl of reaction solution for 60 min at 37°C before reading absorbance at 450 nm. A standard curve was prepared from serial dilutions of glucose with double distilled water (20-100 mg/dL). Intra- and inter-assay coefficients of variation were 4.1 and 9.2%. All assays were read by spectrophotometer (Synergy HT; BioTek Instruments, Winooski, VT).

Statistical Analyses

All component yields were calculated per quarter by multiplying the component percent by the quarter milk yield. For foremilk samples, concentrations of protein and

lactose were calculated as the percentage in fat-free milk according to the following equation: [X/(100-fat)] x 100%, where X is the concentration of protein or lactose, and fat is the fat concentration for a given sample. Covariate values for rectal temperature, milk yield, and component-related variables were then calculated as means of -24 and 0 h. Lactate, MUN, and blood constituents were not measured at -24 h and thus covariates were based on 0 h values.

Data were analyzed for normal distributions and outliers beyond 3 standard deviations were removed; SCC and lactate data were $\log_{10}$-transformed. Data were analyzed as a randomized complete block design in SAS 9.4 (SAS Institute, Inc.) using the PROC MIXED procedure with repeated measures. For rectal temperature and blood constituents, the statistical model included fixed effects of treatment, time, the interaction of treatment and time, and the pre-infusion covariate, with block as the random effect. Cow within treatment was the subject for repeated measurements over time. The covariance structure selected was first order autoregressive. For all milk data, the statistical model was slightly modified with quarter as the experimental unit and random effects of cow over time within block. The covariance structure selected was compound symmetry. For variables with missing data, degrees of freedom were calculated using the Kenward-Roger approximation method. When main effects or interactions were significant, differences between means were determined using the PDIFF option. Covariate-adjusted least-squares means and standard errors of the means are reported. Significance was declared at $P < 0.05$.

Results

By 3 h post-infusion, T cows developed hyperthermia ($P < 0.01$), which persisted for at least three hours and had resolved by 12 h (Figure 2.2). The following morning (24 h), T cows had lower rectal temperatures compared to C cows ($P < 0.05$). Temperatures in C cows remained stable over the duration of the experiment.

Milk Production

Prior to infusions, quarter-level milk yield did not differ between cow treatment groups (T vs C; $P > 0.25$) or udder-halves ($P = 0.12$). Individual front quarter milk yield (12 h interval) for all treatments averaged 3.14 ± 0.27 kg per quarter at 0 h. To assess potential effects of saline infusion on mammary function, we compared C quarters, C-S and C-N. Overall milk yield in C-N was lower than C-S (Table 2.1) because yields were numerically lower throughout the experiment and significantly lower ($P = 0.03$) at 12 h.

Overall milk yield was lower in T than C cows (Table 2.1; $P < 0.05$). At 12 h, milk yields of T-L and T-S quarters were lower than their pre-infusion yields ($P = 0.04$) but not different from yields of C quarters (Figure 2.3). By 24 h, yields of T-L and T-S quarters were less than yields of C quarters (Figure 2.3; $P < 0.001$).

Harvested Milk. These samples were obtained from machine-milkings at normal 12 h intervals. By 12 h post-infusion, milk somatic cell concentration was substantially higher in T-L compared to T-S and C quarters ($P < 0.001$) and remained elevated at 24 h, with most samples from T-L quarters exceeding the maximum detection limit of 10 million cells/mL. On the other hand, there was no difference between quarters in fat, total protein, or lactose concentration of milk obtained at these milkings (Table 2.1).

Yield of somatic cells was higher in T-L than T-S and C quarters at 12 and 24 h milkings ($P < 0.001$), but yield of T-S quarters did not differ from C quarters until 24 h, when T-S yield was lower (Table 2.1; $P = 0.01$). Compared to 12 h, both T-L and T-S quarters yielded fewer cells at 24 h, concomitant with the further decline in milk yield. In comparison, all yields of fat, protein, and lactose in both T-L and T-S declined over time, becoming significantly different to both C quarters at 24 h (Table 2.1; $P < 0.01$).

Foremilk Components

Somatic Cells. SCC increased over 30-fold in LPS treated quarters by 6 h (Figure 2.4A). At 3 h, SCC in foremilk increased in all quarters except T-S ($P < 0.01$), before C quarters began to return to pre-infusion levels. By 6 h, SCC in T-L was higher than all other quarters ($P < 0.001$). The SCC in T-S quarters was not different from C quarters, though it was numerically higher by 12 h.

Lactate. Milk lactate concentration reflected the increased SCC in T-L quarters (Figure 2.4B). By 6 h, lactate in T-L quarters increased 6-fold from 0 h ($P < 0.001$). Lactate continued to rise in T-L for the remainder of the experiment. Lactate concentration in T-S was not different from C quarters.

Fat. Foremilk samples had lower fat concentrations than harvested samples at 0 h (2.2 ± 0.31 vs $3.8\% \pm 0.21$, $P < 0.01$). Unlike harvested samples, a treatment by time interaction (Figure 2.4C; $P = 0.03$) in foremilk revealed treatment differences at 3 and 6 h. Compared to 0 h, milk from both C quarters had higher fat concentration at 3 and 6 h with roughly 3- and 2-fold increases, respectively. In contrast, the normal increase in fat concentration was almost entirely blocked in both T-L and T-S, being lower ($P < 0.05$)

than C quarters at 3 and 6 h. By 12 h, fat concentration returned to 0 h levels with no difference between treatments.

Protein. A treatment by time interaction ($P < 0.01$) for protein concentration indicated a transient effect on T-L quarters at 3 h (Figure 2.4D). Compared to 0 h, all quarter treatments except T-L had lower protein concentration at 3 h ($P < 0.01$), whereas T-L quarters declined more gradually and were not lower than pre-infusion until 12 h. Although there was no difference between treatments after 3 h, treatments evoked different patterns of response in protein concentration: C quarters increased to near pre-infusion levels by 12 h, T-S quarters remained unchanged from 3 h on, and T-L quarters decreased, reaching T-S levels by 12 h.

Lactose. In contrast to fat and protein, lactose concentration of foremilk decreased in T-L and T-S over time to different degrees (Figure 2.4E). An interaction between treatment and time ($P < 0.001$) revealed distinct changes in lactose concentration within and across treatments (Figure 2.4E). In C quarters, lactose fell slightly between milkings ($P < 0.05$) before returning to pre-infusion levels at 12 h. In T-L and T-S, lactose concentration fell below pre-infusion levels ($P < 0.01$) at 6 h in T-L quarters, preceding the drop in T-S quarters at 12 h. Lactose concentration from both T-L and T-S was lower than C quarters by 12 h ($P < 0.05$) and was further reduced by 24 h. In addition, lactose remained lower in T-L compared to T-S quarters from 6 h on; the difference reaching significance at 24 h ($P < 0.01$).

Urea Nitrogen

Concentrations of BUN and MUN increased over time (Figure 2.5, $P < 0.001$). Compared to pre-infusion levels, MUN increased earlier in C than T cows (6 vs 24 h; $P <$

0.001). This followed changes in BUN concentrations, as results indicated a positive relationship between BUN and MUN (r = 0.84, $P < 0.001$) but no interaction between treatment and time for BUN ($P = 0.14$) or MUN ($P = 0.10$). In comparing the ratio of quarter MUN to BUN, there was a time effect but not a treatment effect, with the greatest difference between milk and blood at 3 h post-infusions.

Plasma Components

Plasma glucose concentration in T cows was not different from C cows through 6 h, but then declined from 6 to 12 h (Figure 2.6A; $P < 0.001$) before returning to pre-infusion levels by 24 h. Likewise, the antioxidant potential of plasma was lower in T compared to C cows at 6 and 12 h (Figure 2.6B; $P < 0.01$) but was no longer different by 24 h.

Discussion

The collection of two types of milk samples, coupled with the unilateral treatments, enabled us to identify distinct local and systemic responses to LPS. Our results agreed with previous studies, wherein LPS-treated cows had significantly elevated SCC only in LPS-infused glands (Guidry et al., 1983; Shuster et al., 1991a; Mehrzad et al., 2001) but milk yield and components were depressed in both infused and non-infused quarters (Shuster et al., 1991a). Foremilk samples in particular revealed time-dependent effects of treatment on components, corroborating a prior report of lower fat and lactose concentrations at 6 h (Shuster et al., 1991a). Further, changes in protein concentration of milk from LPS-infused glands preceded the increase in SCC (Guidry et al., 1983; Wellnitz et al., 2015). As a first step toward identifying the underlying mechanisms, we

identified several variables that were affected by local or systemic effects and improved the temporal resolution of acute responses.

Our collection of foremilk was designed to allow analysis of an acute time course while mitigating effects on 12 h milk yields. By not fully milking quarters at 3 and 6 h, we avoided changes in composition due to frequent milk removal (Linzell, 1967) at the expense of determining yields at those times. This sampling design proved successful, as LPS treatment affected all components in foremilk. Foremilk fat and protein concentration responded the earliest, at 3 h post-infusion, before subsequent changes in SCC and lactose at 6 h. However, unlike protein, fat was affected not only in LPS-infused but also the contralateral saline-infused glands; we will refer to the effect on these saline-infused glands as the systemic response. When a component was only affected in LPS-infused glands, e.g. SCC, or affected to a greater extent in the LPS-infused glands, e.g. lactose, we will refer to this as the local response.

Because fat concentration is known to be higher in hind and residual milks (Nielsen et al., 2005), milk collected between milkings was expected to have a higher fat concentration, as observed in control cows (Figure 2.4C). However, this normal increase appeared to be blocked by a systemic effect. This effect must be rapid to affect cisternal milk present in the udder after milking. Further, the effect should be reversible, as fat yields rebound to pre-infusion levels sooner than protein and lactose without evidence of hypersecretion (Shuster et al., 1991a). The simplest explanation, dilution, was not supported by our data as neither protein nor lactose showed a simultaneous systemic change in concentration. We therefore suggest three possible mechanisms: increased lipolysis, suppressed release of milk fat globules (**MFG**) from the apical membrane, or

stronger adhesion of MFG to each other or to the luminal epithelium. Of these, only lipolysis has been studied, though increased concentrations of free fatty acids in milk were attributed more to increased influx from blood or mechanical damage post-harvesting than catabolism of MFG (Needs and Anderson, 1984). If there is a post-secretory effect on milk fat, then the decline in fat yields over 24 h may not be due solely to reduced synthesis or secretion from alveoli, as previously interpreted (Shuster et al., 1991a). Given the nature of the systemic effect, further studies designed to analyze all aspects of milk fat, from synthesis to post-secretion, are warranted to understand the regulation of milk fat synthesis and secretion during mastitis.

In contrast to fat, our results suggest that protein concentration was primarily regulated by local effects. This was mainly consistent with previous findings (Guidry et al., 1983; Shuster et al., 1991a), though the latter also found systemic regulation of protein concentration. Leakage of albumin and transport of immunoglobulins across the blood-milk barrier occurs in LPS-infused glands by 2-4 h post-infusion and before the rise in SCC (Guidry et al., 1983; Wellnitz et al., 2015). However, the subsequent increased migration of polymorphonuclear neutrophils may balance protein influx with proteolysis (Mehrzad et al., 2005). These changes are consistent with our results regarding timing and pattern of changes in protein concentration relative to SCC.

Compared to fat and protein, LPS effects on lactose concentration were delayed and this was true of both local and systemic effects. As the main determinant of milk volume, lactose is the least variable component in terms of concentration. For lactose concentration to decrease, there must be a rise in other osmotically-active molecules, such as electrolytes (Stelwagen et al., 1999; Bruckmaier et al., 2004). Guidry et al. (1983)

reported similar local and systemic reductions in lactose concentration, which generally correlated with increased Na^+ and Cl^- concentrations. What remains unknown is the cause of the reduced lactose, reflected in lower yields at milking. Lactose is known to leak into blood (Stelwagen et al., 1994) and be excreted in urine (Fetherston et al., 2006) when the integrity of the blood-milk barrier is compromised. On the other hand, a systemic effect on lactose synthesis cannot be ruled out. In cultured murine mammary epithelial cells, addition of inflammatory cytokines known to respond to LPS negatively affected the lactose synthesis pathway (Kobayashi et al., 2016). While both may explain our responses in lactose yield, this experiment was not designed to test the sources of this effect.

Milk urea nitrogen, a hydrophilic molecule known to readily equilibrate across the blood-milk barrier (Spek et al., 2016), was largely unaffected by LPS. The rise in serum urea nitrogen in all cows likely reflected changes in blood chemistry or urea metabolism possibly due to normal diurnal variation or in response to the stress of biopsies, repeated sampling, and, in the case of treated cows, altered feed intake. The apparent delay in equilibration of MUN to BUN concentrations is in accordance with the urea flux model proposed by Spek et al. (2016), which predicts a period of 4 h for a difference of 3.5 mg/dL to disappear. For LPS to affect MUN, levels should equilibrate either faster or slower than predicted within the same cow. It is interesting that our results appear to show that at 3 h, when the ratio of MUN to BUN was lowest, LPS-infused quarters had the highest ratio, suggesting faster equilibration.

Although components were initially regulated differently in response to LPS, yields of milk, lactose, protein, and fat were all systemically reduced by the first milking

post-infusions. This confirms that a systemic factor(s), released into blood from either the LPS-infused gland or from non-mammary tissue, causes hypogalactia. In fact, infusing LPS intravenously also induced an overall reduction in milk yield and components without triggering an influx of leukocytes (Shuster et al., 1991b), confirming that local mammary inflammation and elevated SCC is not required to elicit this response. Rather than acting via post-secretory effects, such as leakage through the blood-milk barrier or destruction of components, some have proposed that this systemic factor reduces the synthetic or metabolic capacity of the gland (Shuster et al., 1991a; Silanikove et al., 2011; Kvidera et al., 2017). We therefore chose three theories to test.

We measured antioxidant potential by FRAP to determine if systemic oxidative stress could reduce the synthetic capacity of the mammary gland. Oxidative stress results from an excess of reactive oxygen species (**ROS**) relative to antioxidants and has been associated with cellular and lipid damage (Sordillo and Aitken, 2009). Mehrzad et al. (2001) reported that PMN generate more ROS in the first 12 h following activation by LPS, which coincides with our finding of lower antioxidant potential from 6 to 12 h post-LPS infusion (Figure 2.6B) while SCC was elevated locally. Although FRAP alone cannot confirm oxidative stress, intramammary LPS might cause the release of ROS into circulation prior to systemic effects on mammary function.

Another proposed cause of hypogalactia is a limited availability of milk precursors, particularly glucose. Although our transient drop in blood glucose concentration agrees with Waldron et al. (2003) and may explain lower milk yields at 12 h, it did not correlate to changes in composition or the lowest yields at 24 h. Moreover, Kvidera et al. (2017) reported that infusing glucose to maintain euglycemia did not rescue

milk production in an LPS-infusion study, casting doubt on blood glucose concentration controlling milk synthesis. Instead, this could imply a lower mammary extraction of glucose from blood, which would explain why glucose concentrations had returned to normal at the time of most severe hypogalactia.

Rather than nutrient availability or extraction directly limiting production, Silanikove et al. (2011) proposed that the mammary gland switches to glycolysis during mastitis, thereby utilizing less energy for milk production and conserving glucose for immune function. They theorized that increasing levels of L-lactate in milk indicated that mammary epithelial cells had shifted to glycolytic metabolism (Silanikove et al., 2011). However, we observed an increase in lactate only in milk from LPS-infused glands despite the systemic reduction in milk production and component synthesis, so this theory clearly cannot account for systemic effects. Furthermore, increased milk lactate concentrations have been associated with elevated SCC (Davis et al., 2004), presumably because neutrophils rely on glycolysis for energy metabolism and generate lactate as the end product (Borregaard and Herlin, 1982). Thus, while we cannot exclude the possibility that epithelial cells undergo altered metabolism during mastitis, our milk lactate results do not support a switch to glycolysis.

Several previous studies and lines of evidence suggested that cytokines elicited by LPS could function as mediators of local and systemic effects (Shuster et al., 1993; Persson Waller et al., 2003; Vernay et al., 2012). Indeed, the changes we and others observed in milk yield and components coincide with or follow changes in the expression and concentrations of various cytokines. Leukocyte-recruiting interleukins (IL-1, -6 and -8) can increase markedly in challenged glands by 2 h (Shuster et al., 1993; Persson

Waller et al., 2003) which may explain the subsequent local rise in SCC. Different cytokines also cause different effects. For example, lactose synthesis-related genes may be strongly down-regulated by one cytokine but differentially-regulated by another (Kobayashi et al., 2016). Cytokines are also not limited to local quarters, because as concentrations of IL-8 in milk rose, a small but simultaneous increase occurred in both afferent and efferent lymph (Persson Waller et al., 2003), potentially affecting neighboring quarters. Our results show that each major milk component responded distinctly, in terms of local, systemic, and temporal effects, implying the action of a single mediator on different pathways, differences in sensitivity between pathways, or, most likely, multiple mediators that act uniquely on milk components but collectively cause an overall reduction in milk yield.

Conclusions

All major milk components were regulated uniquely, responding to local or systemic effects within the first 12 h after unilateral LPS infusion. Changes in milk fat concentration were the first indication of systemic effects, which occurred within 3 h of LPS-infusion. Changes in antioxidant capacity, plasma glucose, and lactate did not account for the systemic suppression of mammary function. We conclude that different mediators exert unique effects on milk components during the acute response to LPS.

Table 2.1. Effects of intramammary LPS, saline, or no infusion on milk components from harvested samples at 12 and 24 h[1]

		Treatment[2]					*P*-value		
Item[3]	Time	T-L	T-S	C-S	C-N	Pooled SEM	Trt	Time	Trt x Time
Milk, kg	12 h	2.10[b]	1.90[b]	2.74[a]	2.29[b]	0.28	0.004	<0.001	0.06
	24 h	0.96[b]	1.18[b]	2.95[a]	2.73[a]				
Fat, g	12 h	85.5	81.4	113.1	98.5	16.0	0.05	0.07	0.27
	24 h	36.1[b]	45.0[b]	108.3[a]	106.7[a]				
Protein, g	12 h	65.7[b]	58.4[b]	86.8[a]	72.9[b]	9.1	0.005	<0.001	0.11
	24 h	32.3[b]	43.6[b]	91.2[a]	85.5[a]				
Lactose, g	12 h	90.4[b]	88.6[b]	127.3[a]	105.0[b]	12.1	0.001	<0.001	0.03
	24 h	43.2[b]	51.6[b]	135.7[a]	125.4[a]				
SCC[4], cells	12 h	10.14[a]	8.42[b]	8.45[b]	8.59[b]	0.16	<0.001	0.001	<0.001
	24 h	9.65[a]	8.16[c]	8.73[b]	8.87[b]				
Fat, %	12 h	4.45	4.73	4.03	4.01	0.65	0.36	0.01	0.14
	24 h	5.97	5.78	3.69	3.78				
Protein, %	12 h	3.20	3.14	3.25	3.22	0.20	0.56	0.30	0.30
	24 h	3.83	3.98	3.21	3.18				
Lactose, %	12 h	4.34	4.65	4.63	4.60	0.09	0.49	0.16	0.08
	24 h	4.39	4.36	4.60	4.59				
SCC[4], cells/mL	12 h	6.83[a]	5.16[b]	5.04[b]	5.25[b]	0.14	<0.001	<0.001	<0.001
	24 h	6.91[a]	5.32[b]	5.30[b]	5.45[b]				

[a,b]Covariate-adjusted LSmeans within row with different superscripts differ (P < 0.05).
[1]Data are covariate-adjusted LSmeans ± pooled SEM. Covariates calculated as described in methods
[2]Quarter treatments: T-L = treated cow, LPS-infused quarter (50 μg LPS/10 mL saline); T-S = treated cow, saline-infused quarter (10 mL saline); C-S = control cow, saline-infused quarter (10 mL saline); C-N = control cow, non-infused quarter.
[3]Per front quarter at regular machine milkings (12 h milking interval).
[4] SCC data $\log_{10}$ transformed.

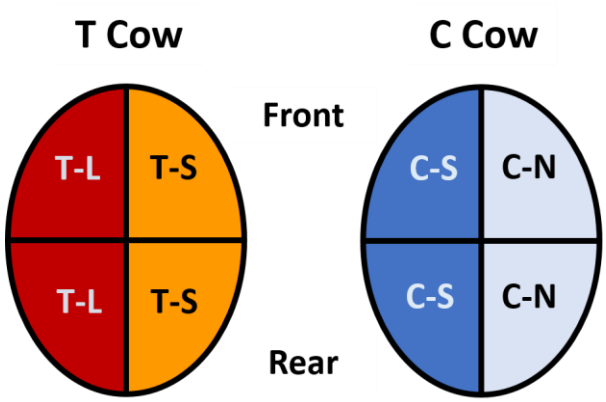

Figure 2.1. Experimental design. Cows were paired (see methods) and one cow of each pair was randomly assigned to receive intramammary infusions of LPS in both quarters of one udder-half. These cows were designated (T) to indicate LPS treatment. Quarters of T cows that were infused with LPS (50 µg in 10 mL saline) were designated (T-L) and their contralateral quarters, that received 10 mL saline, were designated (T-S). The other cow of each pair was designated as control (C). Udder-halves of C cows either received infusions of 10 mL saline (quarters designated C-S) or no infusions, designated (C-N).

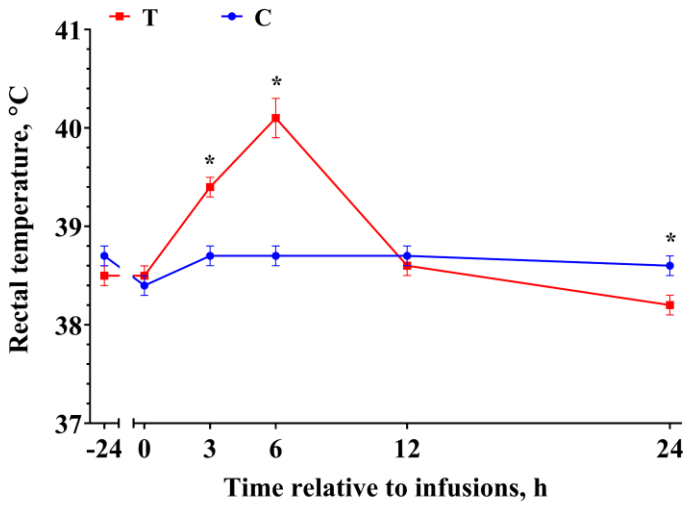

Figure 2.2. Rectal temperature relative to LPS infusion in treated (T) or control (C) cows. LSmeans ± SEM. Treatment x time effect, $P < 0.001$. *Means within time differ significantly $P < 0.05$.

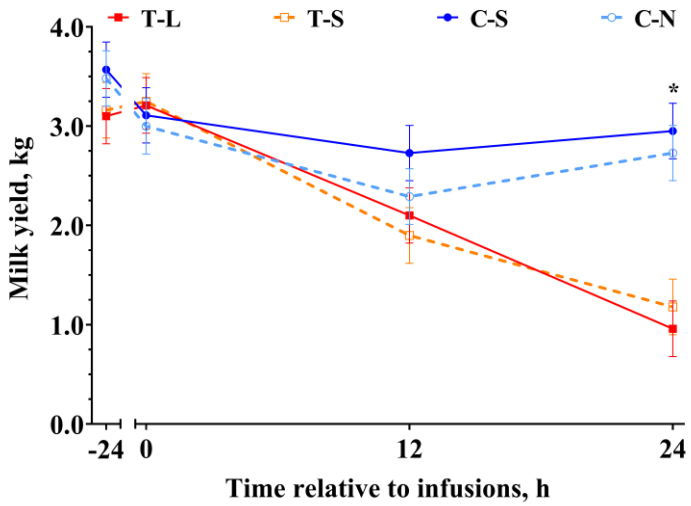

Figure 2.3. Front quarter milk production at milkings relative to treatment: control, no infusion (C-N) or saline (C-S); treated, 50 µg LPS (T-L) or saline (T-S). LSmeans ± SEM. Treatment effect, $P < 0.01$. *Means within time differ significantly between cow treatment $P < 0.05$.

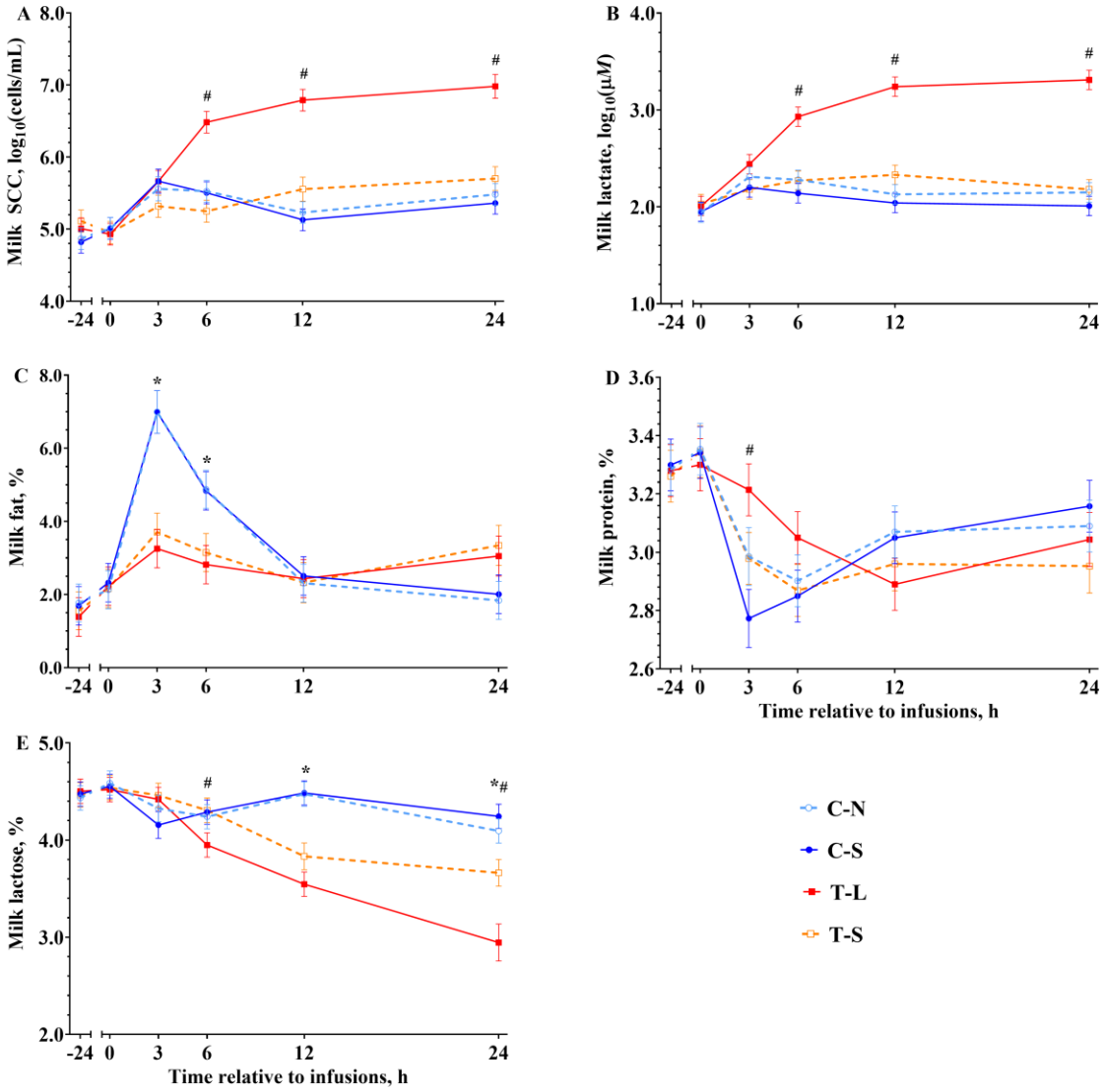

Figure 2.4. Foremilk concentration of A) SCC, B) L-lactate, C) fat, D) protein, and E) lactose, relative to treatment: control, no infusion (C-N) or saline (C-S); treated, 50 μg LPS (T-L) or saline (T-S). SCC and lactate log_{10}-transformed. LSmeans ± SEM. A-E: treatment x time effects, $P < 0.05$. Within time, significant difference of $P < 0.05$ indicated by * between cow treatment and # between T-L and T-S.

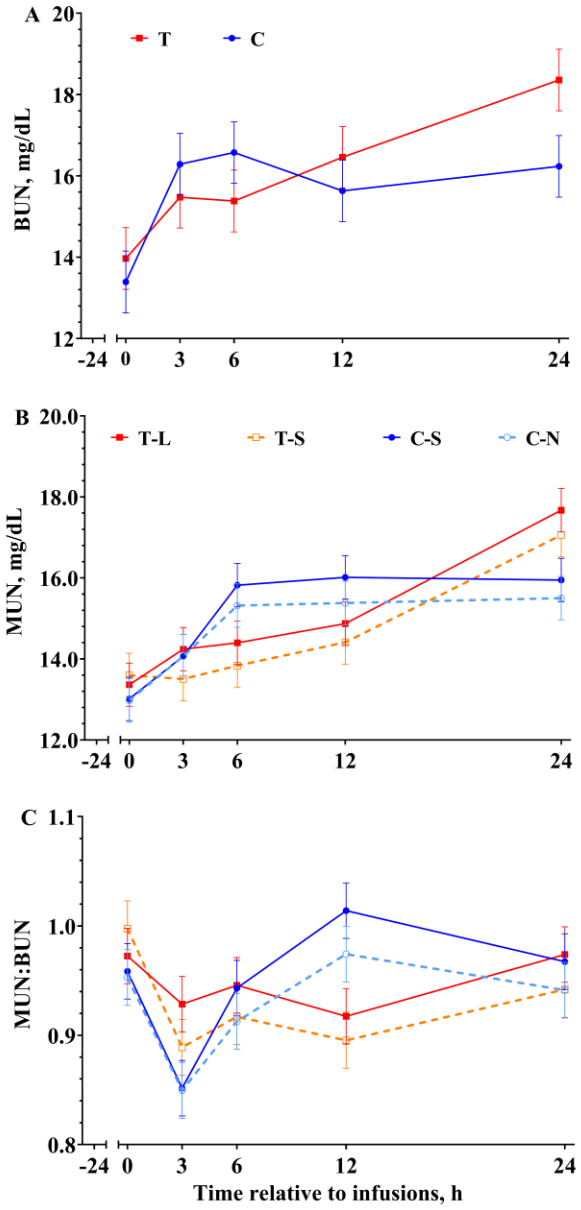

Figure 2.5. Urea nitrogen concentrations in A) serum, and B) foremilk, and C) the ratio of quarter MUN to BUN, relative to LPS infusion. Cow treatment: treated (T) or control (C). Quarter treatment: control, no infusion (C-N) or saline (C-S); treated, 50 μg LPS (T-L) or saline (T-S). LSmeans ± SEM. Time effect, $P < 0.01$.

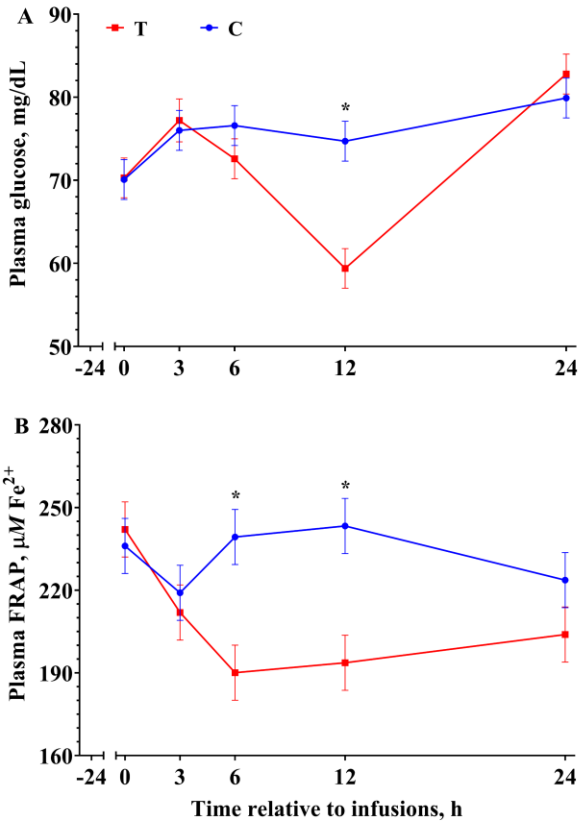

Figure 2.6. Plasma A) glucose and B) antioxidant potential (FRAP) relative to LPS infusion in treated (T) or control (C) cows. LSmeans ± SEM. Treatment x time effects, $P < 0.05$. *Means within time differ significantly $P < 0.01$.

CHAPTER 3: HYPOGALACTIA IN MAMMARY QUARTERS ADJACENT TO LPS-INFUSED QUARTERS IS ASSOCIATED WITH TRANSCRIPTIONAL CHANGES IN IMMUNE GENES

Abstract

Infusion of lipopolysaccharides (LPS) into a mammary gland can provoke inflammatory responses and impair lactation in both the infused gland and in neighboring glands. To gain insight into the mechanisms controlling the spatiotemporal response to localized mastitis in lactating dairy cows, we performed RNA sequencing on mammary tissue from quarters infused with LPS, neighboring quarters in the same animals, and control quarters from untreated animals at 3 and 12 h post-infusion. Differences in gene expression were declared significant at false discovery rate (FDR) $P < 0.05$, log2 fold change > 1.0, and were annotated to KEGG pathways. Comparing mammary transcriptomes from all three treatments revealed 3,088 and 1,644 differentially expressed (DE) genes at 3 and 12h, respectively. Of these genes, > 95% were DE only in LPS-infused quarters and represented classical responses to LPS: inflammation, apoptosis, tissue remodeling, and altered cell signaling and metabolism. Although relatively few genes were DE in neighboring quarters (56 at 3 h; 74 at 12 h), these represented several common pathways. At 3h, TNF, NF-κβ and NOD-like receptor signaling pathways were identified by the upregulation of anti-inflammatory (*NFKBIA, TNFAPI3*) and cell adhesion molecule (*VCAM1, ICAM1*) genes in neighboring glands. Additionally, at 12h, several genes linked to one-carbon and serine metabolism were upregulated. Some responses were also regulated over time. The pro-inflammatory response in LPS-infused

glands diminished between 3 and 12 h, indicating tight control over transcription to reestablish homeostasis; in contrast, two glucocorticoid-responsive genes, *FKBP5* and *ZBTB16*, were among the top DE genes upregulated in neighboring quarters at both timepoints, indicating potential regulation by glucocorticoids. We conclude that a transient, systemic immune response was sufficient to disrupt lactation in neighboring glands, which may be mediated directly by pro-inflammatory factors from the LPS-infused gland or indirectly by secondary factors released in response to systemic inflammation.

Introduction

The bovine mammary gland is regulated by both local and systemic factors during mastitis. At the individual quarter level, a targeted immune response is often required for efficient elimination of opportunistic pathogens. Recognition of pathogen-associated molecular patterns by mammary epithelial cells (**MEC**) and surveilling immune cells triggers the rapid induction of an innate immune response. Consequently, transcriptomic analyses consistently identify a local upregulation of genes encoding pro-inflammatory cytokines, acute phase proteins, and complement factors (Günther et al., 2009; Mitterhuemer et al., 2010; Minuti et al., 2015). Concentrations of cytokines, vasodilators, antimicrobial proteins, and phagocytic immune cells subsequently increase (Bouchard et al., 1999; Mehrzad et al., 2001; Persson Waller et al., 2003). However, this immune response is not exclusive to the inflamed quarter. Mastitis provokes several systemic effects, including fever, elevated plasma cytokines (Persson Waller et al., 2003), and the production of acute phase proteins in the liver (Suojala et al., 2008; Moyes et al., 2016). Moreover, quarters neighboring inflamed glands show enhanced expression of protective

immune genes (Mitterhuemer et al., 2010; Jensen et al., 2013) which may limit bacterial multiplication in the event of a subsequent infection (Suojala et al., 2008).

Notably, the systemic effects of mastitis are not restricted to immune responses. When one gland responds to a source of inflammation, lactation can be compromised in all glands, resulting in lower milk yields and altered concentrations of milk components (Shuster et al., 1991a; Paixão et al., 2017; Shangraw et al., 2020). Even when all glands are healthy, hypogalactia and compositional changes result from intravenous infusion of lipopolysaccharide (LPS), a potent endotoxin (Shuster et al., 1991b). Several mechanisms have been proposed to explain the dysregulation of lactation in inflamed quarters, including apoptosis of MEC (Long et al., 2001), loss of epithelial integrity (Lehmann et al., 2013), and the direct antagonism of milk synthesis and secretion (Kobayashi et al., 2013; Silanikove et al., 2016), yet it remains unclear how factors involved in systemic inflammation control lactation in non-inflamed glands.

Based on our previous results, the differential regulation of milk components depends on the quarter and time after LPS infusion, implying that different mediators exert unique effects on lactation (Shangraw et al., 2020). We hypothesized that changes in the mammary transcriptome would reveal potential regulatory mechanisms underlying the observed impacts of LPS-mediated inflammation on milk production and composition. The objectives of this experiment were, first, to determine how gene expression in LPS-challenged and neighboring glands changes over time with respect to milk production; and second, to identify systemically-regulated mechanisms that affect the neighboring glands.

Materials and Methods

Animals and Design

All procedures involving animals were approved by the University of Missouri Institutional Animal Care and Use Committee (Protocol #9283). Full details on experimental design and animal management were recently reported (Shangraw et al., 2020). Briefly, 10 multiparous cows were blocked into pairs and randomly assigned to one of two treatments: 1) LPS treatment (**T**) or 2) control (**C**). Within cow, udder-halves were randomly assigned to one of two sub-treatments. For T cows, ipsilateral front and rear quarters were treated with 50 µg LPS (*E. coli* serotype O55:B5) in 10 mL 0.9% saline (**T-L**), whereas contralateral quarters were treated with 10 mL saline (**T-S**). For C cows, ipsilateral quarters in one half-udder were treated with 10 mL saline (**C-S**) whereas the contralateral quarters were not infused.

Cows were milked twice daily. On the morning of infusions, rear quarters were biopsied for mammary tissue collection and front quarters remained unbiopsied for milk sample collection.

Mammary Biopsies

After the morning milking on the initial day of the experiment, cows were restrained in a stanchion and sedated by i.v. injection of 20-30 µg/kg BW of xylazine (Akorn Inc., Lake Forest, IL). Following methods described by Farr et al. (1996), biopsy sites at approximately mid-height of both rear quarters were clipped, shaved, and disinfected by alternately scrubbing with 70% ethanol and surgical scrub, rinsing with ethanol. Each biopsy site was line-blocked with 1.5mL lidocaine HCl (VetOne, MWI Animal Health, Boise, ID). A 2.5 cm incision was made through the skin and udder

capsule, then 0.5-1.0 g of mammary tissue was removed using a biopsy tool driven by an electric drill, as described (Wall and McFadden, 2007). Immediately after biopsy, incisions were sutured closed and udder halves were aseptically infused via the teat with LPS or saline, according to the experimental design, with infusions marking time 0 h. At 3 and 12 h post-infusion, cows were again restrained and biopsied as above at sites located ~5 cm dorsal to the previous incision. Within 5 min of collection, 20-30 mg of trimmed mammary tissue was frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

RNA Sequencing

Total RNA from mammary tissue of T-L, T-S, and C-S at both 3 and 12 h post-infusion (n = 5 per group) was extracted using the RNeasy Plus Mini Kit (Qiagen Inc., Valencia, CA). Following manufacturer's instructions, frozen tissue samples were disrupted and homogenized, followed by DNA removal and RNA purification before storage at -80°C in nuclease-free water. RNA concentration was measured by NanoDrop (Thermo Scientific, Waltham, MA) and quality was determined by Fragment Analyzer (Advanced Analytical Technologies Inc., Ankeny, IA). RNA integrity number averaged 7.5 ± 0.7. RNA library preparation and high-throughput sequencing services were performed by the University of Missouri DNA Core Facility using an Illumina TruSeq mRNA kit and sequencing on a NextSeq 500 instrument (Illumina Inc., San Diego, CA), which generated 2 x 75 bp paired-end reads.

Alignment of Sequences and Analysis of Differential Gene Expression

The computation for this work was performed on the high-performance computing infrastructure provided by Research Computing Support Services at the

University of Missouri. Raw sequences (fastq) were subjected to adaptor removal and quality trimming using the paired-end mode in the software tool Trimmomatic (version 0.38; Bolger et al., 2014). Trimming occurred to a quality score of 15 in a 4-base sliding window and minimum read length of 25 bp. Remaining reads were assessed for quality using FastQC, then mapped to the *Bos taurus* genome reference ARS-UCD 1.2 using STAR read aligner (version 2.7.0e; Dobin et al., 2012). Aligned paired and unpaired reads were merged using samtools (version 1.9; Li et al., 2009), before quantifying transcript abundance for each sample using featureCounts (version 1.6.3; Liao et al., 2014).

Differentially expressed (**DE**) genes were detected between treatments within time and within treatments across time using the R package DESeq2 (version 1.22.2; Love et al., 2014) in R version 3.5.2 (R Core Team, 2018). Genes were filtered out if not expressed in at least 5 samples. Statistical models analyzed the effects of treatment at 3 or 12 h and the effects of time within treatment (3 and 12h) with block considered a term in the reduced model. Genes were declared significantly differentially expressed at $\log_2$ fold change $\geq |1.0|$ and false discovery rate (**FDR**), $q < 0.05$.

Functional Annotation

Differentially expressed genes were separated by Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) into common or uniquely regulated sets of genes across time and treatment (Figure 3.1). Functional annotation of genes corresponding to these sets was performed by mapping genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using DAVID 6.8 (Huang et al., 2008). All pathways with EASE scores $P < 0.1$ were examined.

Results

Physiological and Production Responses

Full details on the physiological and milk production responses for non-biopsied front quarters were reported previously (Shangraw et al., 2020). Prior to infusions, milk yield of individual rear quarters averaged 4.23 ± 0.53 kg/ 12 h milking interval with an SCC of 95 ± 24 x 10^3 cells/mL. At 3 h post-infusion, T cows displayed pyrexia. Front quarter foremilk samples from T cows showed depressed milk fat concentration but no difference in milk SCC compared to C cow quarters. By 6 h, T-L but not T-S quarters showed a significant increase in SCC along with lower lactose concentrations. At the first milking 12 h post-infusion, SCC remained locally elevated only in T-L, while lactose content was reduced in both T-L and T-S; T quarter milk yields were reduced relative to pre-infusion but were not significantly different from C quarters. Later, at the second milking 24 h post-infusion, milk yields in T-L and T-S quarters were significantly reduced compared to C quarters, being 23 and 32% of pre-infusion levels, respectively, which marked acute systemic hypogalactia.

Overview of Gene Expression

Sequencing of the libraries (n = 5 per group) yielded 39.2 to 49.3 million quality reads per sample that mapped at a ~94% rate to the *Bos taurus* reference genome. Transcriptomic analysis of mammary tissue from all treatment comparisons at 3 or 12 h post-infusion revealed a total of 3,088 and 1,644 unique DE genes, respectively (Figure 3.2). Broadly, DE genes commonly identified in T-L glands compared to either T-S or C-S were defined as the **local** response, whereas all DE genes identified in T-S compared to C-S were defined as the **adjacent** response of the neighboring gland. Within these

definitions, DE genes were further classified by commonality or uniqueness between treatment comparisons (Figure 3.1). In particular, genes that were DE in both T-L and T-S compared to C-S showed a **systemic** response, wherein both quarters must have been exposed to a common factor.

Comparison at 3 h

At 3 h, the local response to LPS revealed 1,524 DE genes, of which 16 genes also showed differential expression in T-S compared to C-S (Figure 3.2A). Upregulated genes (n=956) were associated with immune signaling, chemotaxis, apoptosis, ribosome biogenesis, MAPK signaling, and insulin resistance pathways. Of the top 20 pathways, TNF signaling was the most overrepresented, along with the cytokine-cytokine, chemokine, Toll-like receptor, and RIG-I-like receptor signaling pathways, and apoptosis (Table 3.1). These pathways represent a core innate immune response common to intramammary *E. coli* infection and LPS infusion. Genes enriching these pathways encode pro-inflammatory cytokines (*CSF3, TNF, LIF, IL6, IL1B*), chemokines (*CCL1, CCL5, CCL20, CXCL2, CXCL8*), cell-adhesion molecules (CAM; *ICAM1, VCAM1*), transcription factors (*NFKB1, JUNB, STAT3*), and caspases (*CASP3, CASP8*), among others. On the other hand, downregulated local DE genes (568) were associated with fatty acid metabolism (*FASN, EHHADH)* and tight junctions (*CLDN8, TJP3*), again representing common pathways differentially regulated in response to localized mastitis. As noted above, these changes coincided with depressed concentrations of milk fat.

In contrast to the local response, there were relatively few DE genes in T-S tissue compared to C-S (n=56; Figure 3.2A, Figure 3.3). Nevertheless, six genes were associated with the TNF signaling pathway, of which four (*CX3CL1, TNFAIP3, NFKBIA,*

and *ICAM*) represented a "graded" systemic response; direct exposure to LPS in T-L quarters caused the greatest increase in expression for these genes whereas an indirect exposure to inflammatory mediators, presumably via systemic circulation, also induced a moderate yet significant response in T-S (Figure 3.4, Table 3.2). Notably, the major cytokines influencing this pathway were DE in T-L but not T-S glands compared to C-S, confirming an indirect response to inflammation in the neighboring glands. Another pathway identified in both the local and adjacent response was p53 signaling. Interestingly, two genes in this pathway, *SESN1* and *GADD45G*, showed a unique adjacent response, being DE only in T-S glands compared to T-L or C-S. Collectively, these results support unidirectional signaling between glands while emphasizing the differential regulation of individual genes within common pathways.

Downregulated genes in the adjacent response did not reveal enriched pathways, although several transcription factors (*GATA2, SOX18, HHEX*), a water transporter (*AQP1*), and apelin receptor (*APLNR*) were identified.

Comparison at 12 h

By 12 h, fewer DE genes were identified, mainly due to a decrease in the number of genes found in the local response to LPS. Of the 538 DE genes comprising the local response in T-L, upregulated genes (n=399) were mostly associated with immune signaling pathways, along with HIF-1 signaling, arginine and proline metabolism, and linoleic acid metabolism (Figure 3.2B). Several cytokines (*IL1B, TNFSF9*) and chemokines (*CCL1, CCL5, CXCL2)* remained DE, whereas others were no longer different (*TNF*) or newly identified (*IL10*). Only 5 of the 139 commonly downregulated local DE genes were associated with an enriched pathway, either xenobiotic or taurine

metabolism. Expanding our criteria to include all downregulated DE genes in T-L compared to either T-S or C-S (n=738) further identified PPAR (*CPT1C, SLC27A1, SLC27A5, FABP5*) and calcium (*OXTR, HTR6*) signaling, among other pathways.

Functional analysis of all 74 DE genes in T-S compared to C-S revealed several metabolic pathways (Table 3.3, Figure 3.3). Unlike at 3 h, genes upregulated in both T-S and T-L compared to C-S (n=21) were no longer enriched for immune pathways, but instead for folate-mediated one carbon metabolism (Figure 3.5). Other upregulated genes included a potassium transporter regulatory subunit (*KCNMB4*) and amino acid transporter (*SLC1A4*). Additionally, genes uniquely upregulated in T-S (n=20) were associated with glycine, serine, and threonine metabolism pathway (*CBS, PSPH*) or no common pathway, including lipin 1 (*LPIN1*) and pseudokinase (*TRIB3*). Of the downregulated genes found in T-S (n=33), *CPT1C*, *APOA1*, and *ACSBG1* were associated with PPAR signaling, which may regulate lipid homeostasis.

Comparison within treatment over time

Most DE genes regulated over time were identified in T-L (n=3,482), followed by T-S (n=466), with relatively few in C-S quarters (n=18) (Figure 3.3). For this analysis, direction of regulation describes the change in expression from 3 to 12 h. In T-L, most genes linked to immune function were downregulated from 3 to 12 h. As noted above, although the expression of these genes was diminished by 12 h, most remained DE in T-L compared to T-S or C-S (Figure 3.4). Other downregulated DE genes in T-L were associated with extracellular matrix interaction, ribosome biogenesis, and second messenger signaling; on the other hand, upregulated DE genes were related to metabolism of amino acids, lipids, and carbon. In comparison, DE genes identified in T-S

also showed downregulated immune pathways during this interval, likely reflecting the decline in T-L glands, whereas regulatory proteins and transporters (*KCNMB4, CAMK2G, KCNN2*) were upregulated.

As the baseline for our comparisons, we assessed the response over time in C-S tissue. Among the eighteen DE genes (Figure 3.3), expression of *IL6* and *CCL8* declined from 3 to 12 h, indicating an initial inflammatory response to biopsy, sedation or saline infusion that subsided over time. At the same time, increased expression of *CYR61, CTGF,* and *ADAMTS1*, which are involved in wound healing, presumably reflects healing of the biopsy sites.

A small proportion of genes in T-L and T-S, relative to C-S, were DE at both timepoints. These genes represented ~17% and 7% of the total DE genes in T-L vs C-S and T-S vs C-S, respectively. In T-L, most of these genes were associated with immune pathways, hence the overlap in significantly enriched pathways at 3 and 12 h. However, the 8 genes DE in T-S at both times did not enrich any pathways. These genes encode proteins (*SESN1, FKBP5, MUCL1*), receptors (*NOD2, LYVE1*), a transcription factor (*ZBTB16),* a pseudogene of HSP 90-α (*LOC781339*), and an uncharacterized non-coding RNA (*LOC100847981*).

Discussion

The udder undergoes spatiotemporal changes during a localized episode of mastitis. Effects related to immune function, such as elevated milk SCC, are specific to the inflamed or challenged gland, i.e. local response, yet lactation can be affected in all glands through a systemic effect (Shuster et al., 1991a; Shangraw et al., 2020). Although various transcriptomic studies have captured the immune response of the mammary gland

to mastitis, the mechanisms regulating lactation, both locally and systemically, remain elusive (Mitterhuemer et al., 2010; Jensen et al., 2013; Kobayashi et al., 2013). In agreement with previous results, most DE genes were only identified in the LPS-challenged (T-L) gland, representing the greater local impact of intramammary LPS on the mammary transcriptome. However, by capturing the transcriptomic response of neighboring (T-S) glands, we show that: (1) unidirectional signaling between glands exists prior to the decline in milk yield, (2) a transient inflammatory response provokes delayed effects not only on gene expression but also lactation in neighboring glands, and (3) other, non-mammary sources affected by systemic inflammation may contribute systemic factors that alter lactation.

The top pathways induced in LPS-challenged quarters confirmed the induction of a local, pro-inflammatory immune response. The binding of LPS to its pathogen recognition receptor, TLR4, activates nuclear factor-κβ (NFKB) transcription factors and leads to the rapid transcription of a network of pro-inflammatory cytokines. A major source of the transcripts enriching these pathways are the resident MEC, which express TLR4 and can upregulate transcription of most cytokines and chemokines (Ibeagha-Awemu et al., 2008; Günther et al., 2009, 2011). However, MEC are not the sole source of inflammatory mediators. Chemokines released in response to LPS attract circulating leukocytes to the challenged quarters (Zheng et al., 2006); these recruited immune cells represent a significant source of immune-related transcripts (Prgomet et al., 2005). By 3 h, histological sections from challenged, but not neighboring, quarters showed a significant increase in neutrophils, which preceded the detectable increase in milk SCC (Choudhary et al., unpublished). As such, the dynamic, heterogeneous tissue of our T-L

samples represented a pool of all potential signaling molecules which could directly or indirectly affect neighboring glands.

At 3 h, immune signaling pathways were commonly identified in both the LPS-challenged and neighboring glands but a closer inspection of the individual genes enriching these pathways revealed spatial differences in function. Unlike in LPS-challenged glands, genes found differentially expressed in neighboring glands were characterized by protective or anti-inflammatory functions. Two genes upregulated in the TNF signaling pathway, NFKB inhibitor alpha (*NFKBIA*) and TNF-α-induced protein 3 (*TNFAIP3*), encode proteins that suppress or terminate TNF- and NFKB-mediated signaling (Boone et al., 2004). Both are transcribed by activated NFKB as part of the negative feedback response to control inflammation. Another negative feedback inhibitor showing similar changes in expression, suppressor of cytokine signaling 1 (*SOCS1*), regulates several JAK/STAT signaling cytokines and interferons and is necessary to suppress LPS-mediated inflammation (Gingras et al., 2004). In LPS-challenged glands, LPS or any of its downstream inflammatory mediators could act as autocrine or paracrine mediators to induce these inhibitors. In neighboring glands, however, no genes encoding inflammatory mediators were differentially expressed, implying that this negative feedback response was caused by systemic signals originating from outside the gland. Cross-talk between an inflamed mammary gland and the liver has also been proposed (Minuti et al., 2015; Moyes et al., 2016), wherein cytokines identified in mammary tissue were predicted as upstream regulators. Given that cytokines and other mediators expressed in the LPS-challenged quarter must become diluted upon entry into the circulation, we speculate that the graded expression observed in these genes across

treatments is due to a dose effect, where lower concentrations of a systemic factor act on neighboring glands than in glands with mastitis.

In addition to spatial differences, the strength of the inflammatory response was greater at 3 h post-infusion than at 12 h, despite the progressive decline in milk yields. Clearance of LPS from the gland or refractoriness of the resident cells to inflammation may account for this change. The amount of intramammary LPS declines steadily over a 12 h milking period mainly through cellular internalization and enzymatic cleavage rather than escape into blood (Ziv et al., 1976), leaving fewer ligands to bind to TLR4 and trigger immune responses. At the same time, immunocompetent cells become refractory to LPS, as observed by the lower expression of pro-inflammatory cytokines over prolonged or repeated exposures (Paape et al., 2002; Biswas and Lopez-Collazo, 2009; Günther et al., 2012). The conserved kinetics of immune gene activation should theoretically prevent excessive signaling and promote the transition from acute inflammation toward resolution (Hao and Baltimore, 2009). Further, the downregulation of immune genes appears to be independent of the presence of leukocytes in the gland because milk SCC increased substantially between 3 and 12 h. This suggests that an increase in somatic cells is a symptom of inflammation but not the cause of hypogalactia. Indeed, the absolute number of somatic cells in a mammary gland can remain stable while milk yield drops in response to intravenous LPS (Shuster et al., 1991b). Thus, the initial inflammatory response to LPS may have a greater impact on immune-related gene expression and lactation than is currently appreciated.

In our search for possible mechanisms and causative relationships between inflammation and lactation, we confirmed the presence of two putative mechanisms that

affect only infected or LPS-challenged glands. Although these mechanisms cannot explain the systemic effects in neighboring glands, some may account for the commonly observed earlier and greater local effects on milk yield and composition (Shuster et al., 1991b; Hoeben et al., 2000). The first, apoptosis, was enriched at 3 h in challenged glands by the upregulation of several pro-apoptotic genes (*CASP3, CASP8, BID, LIF, OSM, STAT3*). This agrees with previous reports that intramammary LPS upregulated caspase mRNA expression at 2.5 h (Minuti et al., 2015) and 3-6 h post-infusion (Didier and Bruckmaier, 2004). In mice, upregulation of signal transducer and activator of transcription 3 (STAT3) by the cytokines leukemia inhibitory factor (LIF) and oncostatin-M (OSM) promotes apoptosis and tissue remodeling, leading to involution (Hughes et al., 2012). Although a loss of MEC via apoptosis could directly impact the total synthetic capacity of the gland, our results cannot confirm whether, or to what extent, cell death occurred. In fact, several of the genes also identified in the apoptosis pathway are anti-apoptotic (*BIRC2, BIRC3, NGF*) or have context-specific functions. Perhaps more revealing was that, at 12 h, apoptotic genes were no longer identified and were absent in neighboring quarters, despite milk yields declining markedly from 12 to 24 h in both T-L and T-S glands (Shangraw et al., 2020). Thus, apoptotic processes might be activated at certain stages of localized mastitis but are opposed by proliferative signals and resolve as signals of active inflammation diminish. Similarly, the downregulation of fatty acid and lipid metabolism is commonly reported in inflamed mammary tissue (Mitterhuemer et al., 2010; Moyes et al., 2016), of which the earliest difference was detected 4 h after an intramammary LPS infusion (Zheng et al., 2006). Generally, this pathway is enriched for genes with roles in milk fat synthesis, e.g. fatty acid synthase

(*FASN*) and lipoprotein lipase (*LPL*). However, although our gene expression data agreed with these studies, we note that the downregulation of these genes alone does not indicate causation. Indeed, milk fat concentrations appeared to be controlled by post-secretory mechanisms, rather than reduced synthesis, during the initial 12 h period after infusion of LPS (Shangraw et al., 2020). While we cannot rule out reduced synthesis, this indicates that other mechanisms control the concentration of milk fat during mastitis.

To account for systemic changes in lactation, we hypothesized that the mediator would affect downstream genes in both glands. As discussed above, the most likely cause is a cytokine or similar inflammatory mediator. For example, whereas *NFKBIA* and *TNFAIP3* showed a graded systemic response at 3 h, lactose concentrations in milk displayed an opposite, if delayed, response at 12 h, being lowest in T-L but also significantly reduced in T-S (Shangraw et al., 2020). Given the transient nature of cytokine expression in LPS-challenged glands, this suggests inhibition of lactose synthesis. Recent *in vitro* work with murine MEC has shown that TNF-α reduces the expression of glucose transporters and α-lactalbumin, both necessary for the synthesis of lactose (Kobayashi et al., 2016). Although some mastitis and LPS studies show a decrease in mRNA expression of lactose-related genes (Gross et al., 2015; Moyes et al., 2016), our data and others do not (Mitterhuemer et al., 2010; Buitenhuis et al., 2011). Further research is needed to determine if lactose is regulated by an inflammatory mediator.

In addition to direct stimulation of MEC, systemic inflammatory mediators might initially interact with other organs and tissues to release secondary factors. As a result,

both glands would have equal exposure to the factor. In neighboring glands, we identified two potential secondary factors: glucocorticoids and oxidative stress.

At both 3 and 12 h, glucocorticoid-responsive genes, promyelocytic leukemia zinc finger (*ZBTB16*) and FK506 binding protein 51 (*FKBP5*), were upregulated in neighboring glands. Previous studies reported strong upregulation of *ZBTB16* and *FKBP5*, including in neighboring quarters (Mitterhuemer et al., 2010; Jensen et al., 2013) and liver (Moyes et al., 2016). Notably, both have pleiotropic functions. As immunomodulators, these genes encode proteins with opposing functions in the inflammatory response: ZBTB16 acts as a transcriptional cofactor to restrain transcription of cytokines (Sadler et al., 2015) whereas FKBP5 acts as a scaffold protein to maintain transcription by NFKB (Romano et al., 2015) and as a co-chaperone to inhibit glucocorticoid receptor binding (Denny et al., 2000). Additionally, both reduce the phosphorylation of the serine/threonine kinase Akt; ZBTB16 appears to act indirectly (Chen et al., 2014), while FKBP5 again acts as a scaffold protein to promote interaction between Akt and the phosphatase PHLPP (Pei et al., 2009). Among its many interactions, phosphorylated Akt blocks apoptosis (Baxter et al., 2006) and promotes glucose uptake (Wieman et al., 2007). Negative regulation of phosphorylated Akt through these genes could therefore impact mammary function in both inflamed and neighboring glands; however, additional experiments are necessary to test this hypothesis. We note that these genes might have no impact on mammary physiology but may simply indicate a response to stress-induced secretion of glucocorticoids. Indeed, plasma cortisol levels increased within 2 h of an intramammary infusion of LPS (Shuster and Harmon, 1992) and the synthetic glucocorticoid, dexamethasone, can induce similar changes in milk as

those observed during mastitis (Shamay et al., 2000), but it remains debatable whether the endogenous release of glucocorticoids affects lactation during mastitis (van der Kolk et al., 1991; Shuster and Harmon, 1992).

Lastly, one of the few pathways enriched at 12 h in neighboring glands was one carbon metabolism. In addition to synthesizing energy and purines to support cell proliferation, one carbon metabolism generates antioxidants (Ducker and Rabinowitz, 2017). This was of interest because we initially anticipated that oxidative stress would play a role in disrupting lactation, given LPS stimulation induces the generation and release of reactive oxygen species (Bouchard et al., 1999; Mehrzad et al., 2001; Jin et al., 2016) and upregulates genes encoding antioxidants in all quarters (Mitterhuemer et al., 2010). As predicted, the upregulation of cystathionine β-synthase (*CBS*) and the mitochondrial 10-formyl-tetrahydrofolate (THF) dehydrogenase (*ALDH1L2*) followed our detection of reduced plasma antioxidant levels in LPS-treated cows (Shangraw et al., 2020). CBS is the rate-limiting enzyme for entry of homocysteine into the transsulfuration pathway, which ultimately generates glutathione and taurine for protection against reactive oxygen species (McFadden et al., 2020). ALDH1L2 metabolizes 10-formyl-THF to produce NADPH, a cofactor necessary for the regeneration of the antioxidative, reduced form of glutathione (Fan et al., 2014; Piskounova et al., 2015). These results could indicate that neighboring glands were responding to oxidative stress, but like glucocorticoids, further research is necessary to determine if this factor affects lactation.

Conclusions

Our data support the hypothesis that all mammary glands are affected by systemic factors during localized mastitis. The upregulation of inhibitors in neighboring glands suggested that unidirectional signaling may occur between glands through inflammatory mediators. Further, the kinetics of the inflammatory response indicated that acute but transient inflammation in some glands is sufficient to disrupt the mammary transcriptome and lactation in all quarters. Future studies are required to determine the role of cytokines and secondary factors, such as glucocorticoids, in regulating lactation.

Table 3.1. Top KEGG pathways enriched for up-regulated local DE genes (T-L vs T-S or C-S)[1] at 3 h post-LPS challenge

Name	Count	*P*-Value[2]	Genes	FDR[3]
TNF signaling pathway	41	0.00	*TRAF1, TRAF2, TNF, CCL2, PTGS2, CSF1, CXCL3, MMP9, CXCL2, NFKBIA, NFKB1, CX3CL1, CCL5, LIF, BAG4, CASP3, CCL20, CASP7, CASP8, MAP3K8, BCL3, IL1B, PIK3R5, FAS, PIK3R3, ICAM1, IL18R1, CFLAR, IL6, CEBPB, MAP2K3, CREB1, FADD, GRO1, BIRC3, BIRC2, JUNB, RIPK1, TNFAIP3, IKBKB*	0.00
Cytokine-cytokine receptor interaction	47	0.00	*CSF3, TNFRSF6B, IL1R2, CCL3, TNF, CCL2, TNFRSF12A, OSMR, CSF1, TNFSF15, TGFB3, CXCL8, CXCR1, CCL8, CXCR2, CX3CL1, CCL5, IL7R, CCL4, IL12RB2, LIF, CCL20, CXCR4, IL4R, IL1RAP, TNFRSF18, IL15RA, IL1B, IL2RG, FAS, LOC510185, LTB, IL1A, IL18R1, IL6, IL18RAP, IL2RA, CCL19, CD40, TNFSF9, CCL11, IFNAR2, TNFSF13B, CXCL16*	0.00
Chemokine signaling pathway	38	0.00	*CCL1, CCL3, CCL2, CXCL5, NFKBIB, CXCL3, BCAR1, CXCL2, CXCR1, NFKBIA, CCL8, CXCL8, CXCR2, NFKB1, CX3CL1, CCL5, CCL4, SRC, CCL22, CCL20, CXCR4, PIK3R5, GNG4, PIK3R3, GNG5, LYN, NCF1, HCK, CCL19, GRO1, STAT3, CCL11, CRKL, CXCL16, IKBKB*	0.00
TLR signaling pathway	27	0.00	*CCL3, TNF, TLR2, NFKBIA, CXCL8, NFKB1, TLR4, CCL5, CCL4, MAP3K8, TICAM1, CASP8, IL1B, PIK3R5, PIK3R3, IL6, MAP2K3, FADD, CD40, IKBKE, IFNAR2, CD80, IRF7, RIPK1, IKBKB, CD14*	0.00
Apoptosis	20	0.01	*BID, CFLAR, TRAF2, TNF, CYCS, NFKBIA, FADD, NFKB1, BIRC3, BIRC2, CASP3, CASP7, RIPK1, CASP8, PIK3R5, FAS, PIK3R3, IKBKB, NGF*	0.00
RIG-I-like receptor signaling	18	0.01	*TRAF2, IL6, IFIH1, TNF, NFKBIB, CXCL8, NFKBIA, FADD, TRIM25, NFKB1, TANK, IKBKE, ISG15, IRF7, RIPK1, CASP8, IKBKB, DHX58*	0.00

[1]T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow

[2] EASE Score

[3]Benjamini-corrected false discovery rate

Table 3.2. KEGG pathways enriched for DE genes in neighboring glands (T-S vs C-S)[1] at 3 h post-infusion. Log_2 fold change between T-S and C-S for each gene is reported in parentheses

Name	Count	%[2]	*P*-Value[3]	Genes	FDR[4]
TNF signaling pathway	6	0.08	0.00	*VCAM1 (1.61), ICAM1 (1.88), NOD2 (1.51), NFKBIA (1.81), CX3CL1 (2.12), TNFAIP3 (1.96)*	0.00
NF-kappa B signaling pathway	4	0.06	0.00	*VCAM1 (1.61), ICAM1 (1.88), NFKBIA (1.81), TNFAIP3 (1.96)*	0.13
Epstein-Barr virus infection	4	0.06	0.01	*ICAM1 (1.88), CDKN1A (2.07), NFKBIA (1.81), TNFAIP3 (1.96)*	0.17
NOD-like receptor signaling pathway	3	0.04	0.01	*NOD2 (1.51), NFKBIA (1.81), TNFAIP3 (1.96)*	0.23
p53 signaling pathway	3	0.04	0.02	*CDKN1A (2.07), GADD45G (2.42), SESN1 (1.22)*	0.32
HTLV-I infection	4	0.06	0.06	*VCAM1 (1.61), ICAM1 (1.88), CDKN1A (2.07), NFKBIA (1.81),*	0.56
Cell adhesion molecules (CAMs)	3	0.04	0.09	*VCAM1 (1.61), ICAM1 (1.88), CD274 (2.03)*	0.68
Chemokine signaling pathway	3	0.06	0.07	*CCL1 (2.74), NFKBIA (1.81), CX3CL1 (2.12)*	0.48
African trypanosomiasis	2	0.04	0.08	*VCAM1 (1.61), ICAM1 (1.88)*	0.51

[1]T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow

[2] Percent of DE genes identified in pathway

[3] EASE Score

[4]Benjamini-corrected false discovery rate

Table 3.3. KEGG pathways enriched for DE genes in neighboring glands at 12 h (T-S vs C-S)[1] post-infusion. Log_2 fold change between T-S and C-S for each gene is reported in parentheses

Name	Count	%[2]	*P*-Value[3]	Genes	FDR[4]
Up					
Metabolic pathways	9	0.19	0.01	*MTHFD2 (1.33), PRODH (2.27), CYP2B6 (3.11), GALNT15 (3.35), CYP4F2 (3.05), PSPH (2.05), LPIN1 (2.87), CBS (1.39)*	0.38
Arachidonic acid metabolism	2	0.04	0.02	*CYP2B6 (3.11), CYP4F2 (3.05),*	0.25
One carbon pool by folate	2	0.04	0.05	*MTHFD2 (1.33), ALDH1L2 (2.27)*	0.43
Down					
PPAR signaling pathway	3	0.08	0.00	*CPT1C (-1.99), APOA1 (-2.50), ACSBG1 (-2.15)*	0.12
Fatty acid degradation	2	0.05	0.06	*CPT1C (-1.99), APOA1 (-2.50)*	0.59
Fatty acid metabolism	2	0.05	0.07	*CPT1C (-1.99), APOA1 (-2.50)*	0.50
Adipocytokine signaling pathway	2	0.05	0.10	*CPT1C (-1.99), APOA1 (-2.50)*	0.54

[1]T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow
[2] Percent of DE genes identified in pathway
[3] EASE Score
[4]Benjamini-corrected false discovery rate

Gene Set	Regulation	Statistical significance
1	Common local	T-L ≠ T-S and C-S
2	Graded	T-L ≠ T-S ≠ C-S
3	Systemic	T-L and T-S ≠ C-S
4	Unique local	T-L ≠ C-S
5	Unique local	T-L ≠ T-S
6	Unique adjacent	T-S ≠ C-S
7	Unique adjacent	T-S ≠ T-L and C-S

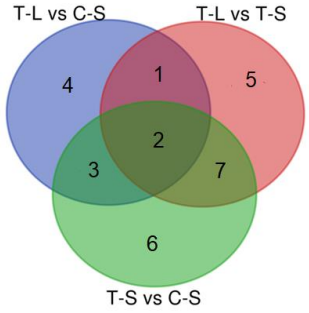

Figure 3.1. Overview of gene sets for comparison of local response to intramammary LPS in challenged gland or adjacent response in neighboring gland. Local response (gene sets 1 and 2) includes all genes DE in T-L compared to T-S or C-S. Adjacent response in neighboring gland (gene sets 2, 3, 6 and 7) includes all genes DE in T-S compared to C-S. Genes DE between treatments (≠) at FDR $q < 0.05$, $\log_2$ fold change ≥ 1.0. T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow.

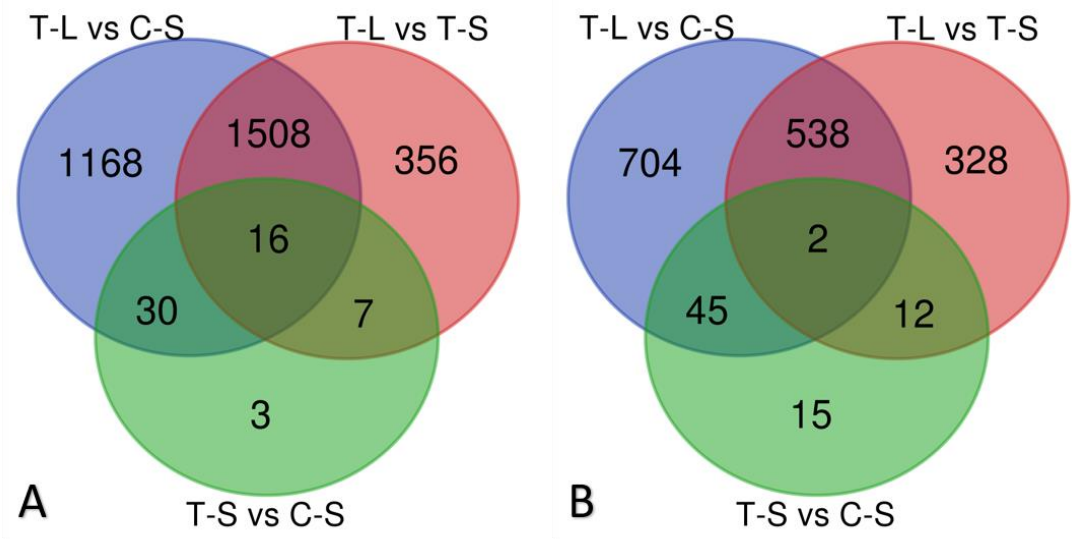

Figure 3.2. Transcriptome analysis of mammary biopsies from LPS-treated and control cows. Total RNA was extracted from five T-L, T-S, and C-S quarters at A) 3 h and B) 12 h post-LPS infusion. Normalized and $\log_2$-transformed read count data and DE genes (FDR, $P < 0.05$) were determined by DESeq2 analysis. Venn diagrams show common and unique DE genes between treatments (see Figure 3.1). T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow.

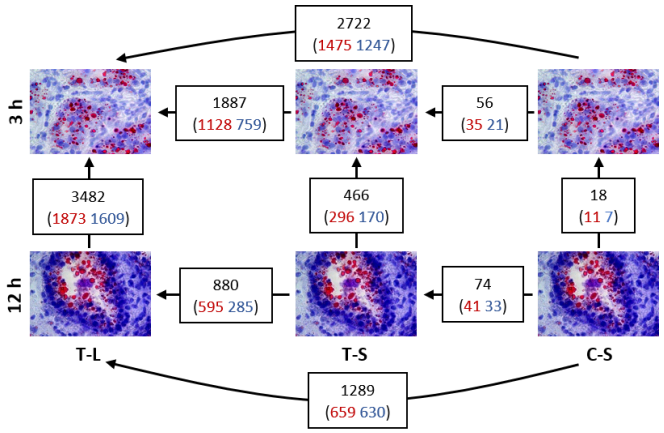

Figure 3.3. Number of genes in mammary tissue DE between treatments at 3 or 12 h after intramammary infusions of LPS or saline. Total number of DE genes for each comparison is shown in black font, with number of upregulated or downregulated genes shown in red or blue font, respectively. T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow. Mammary histology courtesy of R. K. Choudhary.

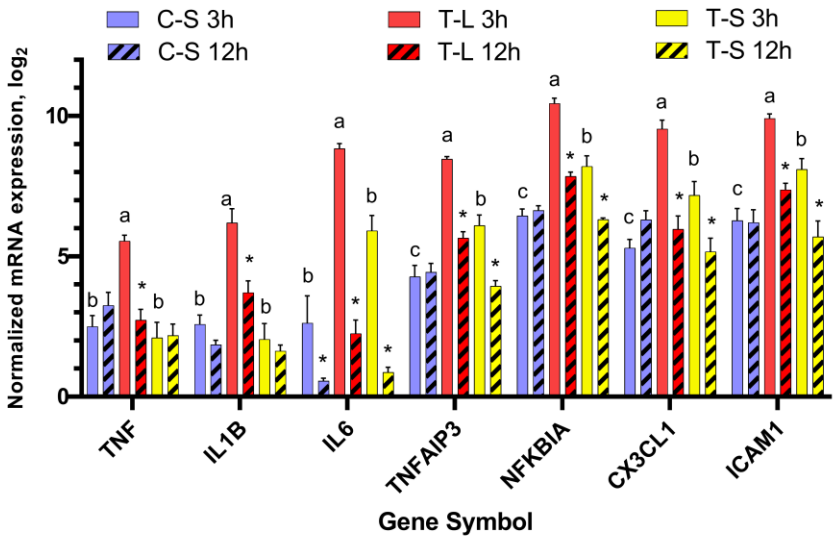

Figure 3.4. Normalized expression of immune genes in the TNF signaling pathway at 3 h post-infusion. Represented genes show local (*TNF*, *IL1B*, *IL6*) or graded (*TNFAIP3*, *NFKBIA*, *CX3CL1*, *ICAM1*) response to LPS-mediated inflammation. T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow. Letters denote significant difference between treatments at 3 h, $q < 0.05$. Asterisks denote significant difference in gene expression between 3 and 12 h within treatment, $q < 0.05$.

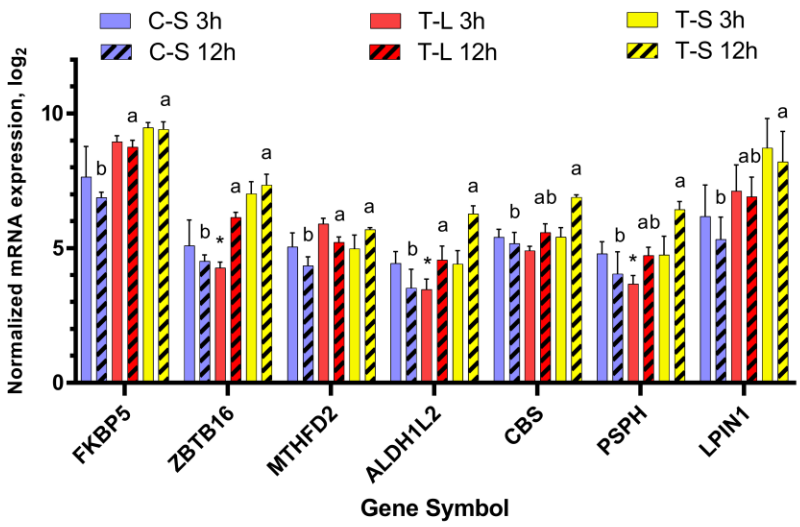

Figure 3.5. Normalized expression of genes DE at 12 h post-infusion in neighboring glands relative to control. T-L: LPS-infused (50 µg) gland of treated cow; T-S: saline-infused gland of treated cow; C-S: saline-infused gland of control cow. Letters denote significant difference between treatments at 12 h, $q < 0.05$. Asterisks denote significant difference in gene expression between 3 and 12 h within treatment, $q < 0.05$.

CHAPTER 4: GENERAL DISCUSSION

The regulation of mammary function during the systemic response to intramammary LPS highlights the multi-factorial nature of inflammation. The earliest responses to intramammary LPS observed in this study supported the systemic action of pro-inflammatory mediators, given the increased expression of immune genes in both challenged and non-inflamed quarters. Subsequent systemic responses indicated that non-inflammatory factors, e.g. glucose, cortisol and oxidative stress, changed as well, which might also have affected mammary function in non-inflamed glands. Collectively, these results emphasize the need to investigate both the primary immune response and secondary responses to inflammation in pursuit of the mechanisms that regulate lactation physiology.

Based on milk production, the systemic response to intramammary LPS induced hypogalactia and exerted unique effects on milk fat and lactose in non-inflamed quarters. Although further research is required to determine the cause of these effects, some factors can be ruled out due to their absence in neighboring glands. One factor that has been misinterpreted as a cause of hypogalactia is milk SCC. Milk SCC is negatively correlated with milk production (Burvenich et al., 2003); however, milk SCC does not need to be elevated in neighboring glands for mammary function to be compromised. Similar conclusions have been reached for milk production (Shuster et al., 1991b; Hoeben et al., 2000) as well as for other physiological outcomes. For example, neutrophil infiltration of intrauterine tissues in response to local inflammation is associated with preterm labor. To determine if inflammation-induced infiltration was necessary to cause preterm labor, mice were depleted of circulating neutrophils prior to an intrauterine LPS challenge

(Rinaldi et al., 2014). Interestingly, the researchers found that preterm labor was not delayed despite the absence of neutrophils in decidual tissues. Thus, the presence of immune cells may be useful as a diagnostic marker but should not be misinterpreted as the cause of a response to inflammation.

Rather than an influx of immune cells, the release of pro-inflammatory mediators into systemic circulation could impact neighboring glands directly or indirectly. The early induction of *TNFAIP3, NFKBIA,* and *SOCS1* in the neighboring glands provides evidence for the direct action of inflammatory mediators throughout the body and was notable for two reasons. First, the increased expression of negative feedback regulators, but not pro-inflammatory cytokines, is consistent with an LPS-sensing mechanism described in macrophages. Low and high doses of LPS can be distinguished by cells, leading to differential expression of genes in the NFKB pathway either to dampen or activate an immune response (Sung et al., 2014). Second, reduced lactose synthesis by mammary tissue and the lower expression of genes related to lactose synthesis have been linked to the NFKB pathway after treatment with LPS or pro-inflammatory cytokines (Kobayashi et al., 2013, 2016). Taken together, these reasons could indicate that different doses of pro-inflammatory mediators alter milk production through direct interactions with MEC, providing an explanation for differences between LPS-infused and saline-infused glands in LPS-treated cows. On the other hand, the direct effects of inflammatory mediators appeared relatively transient in comparison to changes in non-inflammatory factors and the development of hypogalactia. Further investigation is required to determine if the acute, transient action of inflammatory mediators causes hypogalactia, either directly or indirectly. Additionally, how hypogalactia is induced in the non-

inflamed glands remains unknown. The lack of changes in genes related to milk synthesis in non-inflamed glands probably indicates that systemic hypogalactia is not controlled at the level of transcription but instead by metabolic, hormonal, or biochemical changes. Whereas some factors have been controlled during an LPS challenge, i.e. plasma glucose (Vernay et al., 2012; Kvidera et al., 2017) or cortisol concentrations (Shuster and Harmon, 1992), other potential factors released in response to inflammation have not. This presents ongoing challenges for research to distinguish between controlling and confounding factors.

Establishing temporal relationships remains a key element to determining causal mechanisms. Although frequent sampling of foremilk improved the resolution of effects on milk components, milk yields were collected too infrequently to determine the cause of hypogalactia. Because milk must accumulate over time to obtain yields, any factor present prior to the development of hypogalactia could be the regulatory factor. Thus, more frequent milking during systemic inflammation is necessary to determine the underlying physiology and develop effective interventions. The timing of when the regulatory factor acts on mammary tissue may also determine whether hypogalactia can be avoided once an inflammatory state is activated. Sepsis models have shown that appropriate treatments can be ineffective or even harmful if given too late (Tisoncik et al., 2012). The same principle likely applies to milk production: if the determining factor is not opposed or corrected early enough, yields will be reduced until homeostasis is restored.

In addition to more frequent sampling, combining physiological data with global omics data is a powerful method to determine mechanisms. However, collecting samples

to compare these responses has limitations. In this study, biopsies introduced confounding variables, including heterogenous tissue and systemic effects from analgesics and handling stress. The contamination of milk with blood further limited the strength of the analysis, as milk production and gene expression could not be directly compared from the same glands. Isolating RNA from milk fat, as demonstrated by Brenaut et al. (2014), presents a non-invasive method to confirm and extend the data presented here. In comparison to biopsy, milk fat can be collected non-invasively through regular milkings, providing both a milk sample and RNA for further analysis. Additionally, milk fat RNA is derived from MEC cytosol (Masedunskas et al., 2017). Theoretically, this provides a clearer picture of the MEC transcriptome and the secretory capacity of the gland when compared against a random sampling of secretory and stromal cells in mammary biopsies. However, milk fat does have limitations which would need to be accounted for, including contamination by somatic cells and the inability to capture a specific moment in time. Therefore, milk fat should be explored as an alternative to traditional samples to enable more holistic *in vivo* studies of mammary function.

Among the pathways identified by this study, the most interesting was one carbon metabolism because of its link to oxidative stress. One carbon metabolism has been studied extensively in transition and early lactation cows because supplementation of diets with methyl donors improves metabolic health, immune status, and milk production (McFadden et al., 2020). Notably, the peripartal period is also when inflammation and oxidative stress are most prevalent (Bradford et al., 2015). If supplementation reduces inflammation and improves antioxidant status, then feeding methyl donors might reduce the severity of systemic inflammation. However, further research is needed to determine

if oxidative stress, and by extension one carbon metabolism, plays a role in regulating mammary function.

In addition to the regulation of milk production, the initial systemic response to inflammation may confer protection against future insults. The refractory state of the immune response 12 h after LPS infusion suggested the development of a tolerant state. LPS tolerance is hypothesized to limit excessive inflammation while ensuring protection against pathogens through selective feedback mechanisms and epigenetic modifications. Treating mammary glands with low doses of LPS protected those glands against a subsequent *E. coli* infection and significantly reduced clinical symptoms (Petzl et al., 2011). Furthermore, Shuster and Harmon (1991) repeatedly challenged two mammary glands with LPS and reported that milk production partially recovered in challenged glands and fully recovered in neighboring, unchallenged glands. However, it is uncertain whether the systemic response to either LPS or coliform mastitis protects non-inflamed glands from developing an IMI and clinical mastitis. Although transcriptomic evidence from Mitterhuemer (2010) suggested a protective effect, my data showed that by 12 h, immune-responsive genes were no longer differentially expressed in the non-inflamed gland. Moreover, chronically elevated plasma levels of LPS did not protect glands against hypogalactia upon intramammary infusion of LPS (Aditya et al., 2017). This could indicate that i) non-inflamed glands are not exposed to levels of pro-inflammatory mediators necessary to reach a protective threshold, and/or ii) LPS must act directly on epithelial tissue from within the gland to confer tolerance. Therefore, measuring milk synthesis and the immune response of non-inflamed glands after prolonged systemic

inflammation or a subsequent intramammary challenge may help to identify mechanisms of local LPS tolerance and improve prophylactic treatments.

Limiting the effects of systemic inflammation remains a priority to improve productivity and promote health in lactating dairy cows. The influence of inflammation-inducing mediators and secondary metabolic, hormonal, and oxidative responses on mammary tissue remain unclear, but each may contribute to the regulation of milk production. Ultimately, understanding the mechanisms that alter mammary function in non-inflamed glands may lead to breakthroughs in interventions to protect against acute coliform mastitis and its attendant effects on milk production.

REFERENCES

Aditya, S., E. Humer, P. Pourazad, R. Khiaosa-Ard, J. Huber, and Q. Zebeli. 2017. Intramammary infusion of Escherichia coli lipopolysaccharide negatively affects feed intake, chewing, and clinical variables, but some effects are stronger in cows experiencing subacute rumen acidosis. J. Dairy Sci. 100:1363–1377. doi:10.3168/jds.2016-11796.

Aghamohammadi, M., D. Haine, D. F. Kelton, H. W. Barkema, H. Hogeveen, G. P. Keefe, and S. Dufour. 2018. Herd-level mastitis-associated costs on Canadian dairy farms. Front. Vet. Sci. 5. doi:10.3389/fvets.2018.00100.

Aitken, S. L., C. M. Corl, and L. M. Sordillo. 2011. Immunopathology of mastitis: Insights into disease recognition and resolution. J. Mammary Gland Biol. Neoplasia 16:291–304. doi:10.1007/s10911-011-9230-4.

Ayadi, M., G. Caja, X. Such, M. Rovai, and E. Albanell. 2004. Effect of different milking intervals on the composition of cisternal and alveolar milk in dairy cows. J. Dairy Res. 71:304–310. doi:10.1017/S0022029904000329.

Bannerman, D. D. 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87:10–25. doi:10.2527/jas.2008-1187.

Bauman, D.E., and C. L. Davis. 1974. Lactation: A comprehensive treatise. Vol. 2. B. L. Larson and V. R. Smith, ed. Academic Press, Inc., New York.

Baxter, F. O., P. J. Came, K. Abell, B. Kedjouar, M. Huth, K. Rajewsky, M. Pasparakis, and C. J. Watson. 2006. IKKβ/2 induces TWEAK and apoptosis in mammary epithelial cells. Development 133:3485–3494. doi:10.1242/dev.02502.

Benzie, I. F. F., and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70–76. doi:10.1006/abio.1996.0292.

Biswas, S. K., and E. Lopez-Collazo. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 30:475–487. doi:10.1016/j.it.2009.07.009.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

Bonizzi, G., and M. Karin. 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25:280–288. doi:10.1016/j.it.2004.03.008.

Boone, D. L., E. E. Turer, E. G. Lee, R.-C. Ahmad, M. T. Wheeler, C. Tsui, P. Hurley,

M. Chien, S. Chai, O. Hitotsumatsu, E. McNally, C. Pickart, and A. Ma. 2004. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat. Immunol. 5:1052–1060. doi:10.1038/ni1110.

Borregaard, N., and T. Herlin. 1982. Energy metabolism of human neutrophils during phagocytosis. J. Clin. Invest. 70:550–557. doi:10.1172/JCI110647.

Bouchard, L., S. Blais, C. Desrosiers, X. Zhao, and P. Lacasse. 1999. Nitric oxide production during endotoxin-induced mastitis in the cow. J. Dairy Sci. 82:2574–2581. doi:10.3168/jds.S0022-0302(99)75512-8.

Bradford, B. J., K. Yuan, J. K. Farney, L. K. Mamedova, and A. J. Carpenter. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J. Dairy Sci. 98:6631–6650. doi:10.3168/jds.2015-9683.

Brenaut, P., R. Bangera, C. Bevilacqua, E. Rebours, C. Cebo, and P. Martin. 2012. Validation of RNA isolated from milk fat globules to profile mammary epithelial cell expression during lactation and transcriptional response to a bacterial infection. J. Dairy Sci. 95:6130–6144. doi:10.3168/jds.2012-5604.

Brenaut, P., L. Lefèvre, A. Rau, D. Laloë, G. Pisoni, P. Moroni, C. Bevilacqua, and P. Martin. 2014. Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to Staphylococcus aureus. Vet. Res. 45. doi:10.1186/1297-9716-45-16.

Bruckmaier, R. M., C. E. Ontsouka, and J. W. Blum. 2004. Fractionized milk composition in dairy cows with subclinical mastitis. Vet. Med. (Praha). 49:283-290. doi:10.17221/5706-VETMED.

Bruckmaier, R. M., and J. W. Blum. 1998. Oxytocin release and milk removal in ruminants. J. Dairy Sci. 81:939–949. doi:10.3168/jds.S0022-0302(98)75654-1.

Buitenhuis, B., C. M. Røntved, S. M. Edwards, K. L. Ingvartsen, and P. Sørensen. 2011. In depth analysis of genes and pathways of the mammary gland involved in the pathogenesis of bovine Escherichia coli- mastitis. BMC Genomics 12. doi:10.1186/1471-2164-12-130.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet. Res. 34:521-564. doi:10.1051/vetres:2003023.

Capuco, A. V., D. L. Wood, R. Baldwin, K. Mcleod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. J. Dairy Sci. 84:2177–2187. doi:10.3168/jds.S0022-0302(01)74664-4.

Chen, Q., G. He, W. Zhang, T. Xu, H. Qi, J. Li, Y. Zhang, and M.-Q. Gao. 2016. Stromal

fibroblasts derived from mammary gland of bovine with mastitis display inflammation-specific changes. Sci. Rep. 6:27462. doi:10.1038/srep27462.

Chen, S., J. Qian, X. Shi, T. Gao, T. Liang, and C. Liu. 2014. Control of hepatic gluconeogenesis by the promyelocytic leukemia zinc finger protein. Mol. Endocrinol. 28:1987–1998. doi:10.1210/me.2014-1164.

Davis, S., V. Farr, C. Prosser, G. Nicholas, S. Turner, J. Lee, and A. Hart. 2004. Milk L-lactate concentration is increased during mastitis. J. Dairy Res. 71:175-181. doi:10.1017/S002202990400007X.

Denny, W. B., D. L. Valentine, P. D. Reynolds, D. F. Smith, and J. G. Scammell. 2000. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology 141:4107–4113. doi:10.1210/endo.141.11.7785.

Didier, A., and R. M. Bruckmaier. 2004. mRNA expression of apoptosis-related genes in mammary tissue and milk cells in response to lipopolysaccharide challenge and during subclinical mastitis. Milchwissenschaft 59:119–123.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. 2012. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. doi:10.1093/bioinformatics/bts635.

Dosogne, H., E. Meyer, A. Sturk, J. van Loon, A. M. Massart-Leën, and C. Burvenich. 2002. Effect of enrofloxacin treatment on plasma endotoxin during bovine Escherichia coli mastitis. Inflamm. Res. 51:201–205. doi:10.1007/PL00000293.

Ducker, G. S., and J. D. Rabinowitz. 2017. One-carbon metabolism in health and disease. Cell Metab. 25:27–42. doi:10.1016/j.cmet.2016.08.009.

Fan, J., J. Ye, J. J. Kamphorst, T. Shlomi, C. B. Thompson, and J. D. Rabinowitz. 2014. Quantitative flux analysis reveals folate-dependent NADPH production. Nature 510:298–302. doi:10.1038/nature13236.

Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. J. Dairy Sci. 79:543–549. doi:10.3168/jds.S0022-0302(96)76398-1.

Fernandes, A. M., C. A.. Oliveira, and P. Tavolaro. 2004. Relationship between somatic cell counts and composition of milk from individual Holstein cows. Arq. Inst. Biol. 71:163–166.

Fetherston, C. M., C. T. Lai, L. R. Mitoulas, and P. E. Hartmann. 2006. Excretion of lactose in urine as a measure of increased permeability of the lactating breast during inflammation. Acta Obstet. Gynecol. Scand. 85:20-25. doi:10.1080/00016340500324514.

Frost, A. J., A. W. Hill, and B. E. Brooker. 1980. The early pathogenesis of bovine mastitis due to Escherichia coli. Proc. R. Soc. London. Ser. B. Biol. Sci. 209:431–439. doi:10.1098/rspb.1980.0104.

Gingras, S., E. Parganas, A. de Pauw, J. N. Ihle, and P. J. Murray. 2004. Re-examination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling. J. Biol. Chem. 279:54702–54707. doi:10.1074/jbc.M411043200.

Gröhn, Y. T., D. J. Wilson, R. N. González, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87:3358–3374. doi:10.3168/jds.S0022-0302(04)73472-4.

Gross, J. J., H. A. van Dorland, O. Wellnitz, and R. M. Bruckmaier. 2015. Glucose transport and milk secretion during manipulated plasma insulin and glucose concentrations and during LPS-induced mastitis in dairy cows. J. Anim. Physiol. Anim. Nutr. (Berl). 99:747–756. doi:10.1111/jpn.12259.

Guidry, A. J., M. Ost, I. H. Mather, W. E. Shainline, and B. T. Weinland. 1983. Sequential response of milk leukocytes, albumin, immunoglobulins, monovalent ions, citrate, and lactose in cows given infusions of Escherichia coli endotoxin into the mammary gland. Am. J. Vet. Res. 44:2262–2267.

Günther, J., D. Koczan, W. Yang, G. Nürnberg, D. Repsilber, H.-J. Schuberth, Z. Park, N. Maqbool, A. Molenaar, and H.-M. Seyfert. 2009. Assessment of the immune capacity of mammary epithelial cells: Comparison with mammary tissue after challenge with Escherichia coli. Vet. Res. 40. doi:10.1051/vetres/2009014.

Günther, J., K. Esch, N. Poschadel, W. Petzl, H. Zerbe, S. Mitterhuemer, H. Blum, and H.-M. Seyfert. 2011. Comparative kinetics of Escherichia coli- and Staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1a or tumor necrosis factor alpha. Infect. Immun. 79:695-707. doi.org/10.1128/IAI.01071-10.

Günther, J., W. Petzl, H. Zerbe, H.-J. Schuberth, D. Koczan, L. Goetze, and H.-M. Seyfert. 2012. Lipopolysaccharide priming enhances expression of effectors of immune defence while decreasing expression of pro-inflammatory cytokines in mammary epithelia cells from cows. BMC Genomics 13. doi:10.1186/1471-2164-13-17.

Hampton, R. Y., and C. R. Raetz. 1991. Macrophage catabolism of lipid A is regulated by endotoxin stimulation. J. Biol. Chem. 266:19499–19509.

Hao, S., and D. Baltimore. 2009. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat. Immunol. 10:281–288. doi:10.1038/ni.1699.

Hartmann, P. E., and D. S. Kronfeld. 1973. Mammary blood flow and glucose uptake in lactating cows given dexamethasone. J. Dairy Sci. 56:896–902. doi:10.3168/jds.S0022-0302(73)85274-9.

Herve, L., H. Quesnel, V. Lollivier, and M. Boutinaud. 2016. Regulation of cell number in the mammary gland by controlling the exfoliation process in milk in ruminants. J. Dairy Sci. 99:854–863. doi:10.3168/jds.2015-9964.

Hoeben, D., C. Burvenich, E. Trevisi, G. Bertoni, J. Hamann, R. Bruckmaier, and J. Blum. 2000. Role of endotoxin and TNF-α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. J. Dairy Res. 67:503–514. doi:10.1017/S0022029900004489.

Hogeveen, H., W. Steeneveld, and C. A. Wolf. 2019. Production diseases reduce the efficiency of dairy production: A review of the results, methods, and approaches regarding the economics of mastitis. Annu. Rev. Resour. Econ. 11:289–312. doi:10.1146/annurev-resource-100518-093954.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2008. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37:1–13. doi:10.1093/nar/gkn923.

Hughes, K., J. A. Wickenden, J. E. Allen, and C. J. Watson. 2012. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. J. Pathol. 227:106–117. doi:10.1002/path.3961.

Ibeagha-Awemu, E. M., J.-W. Lee, A. Ibeagha E., D. Bannerman D., M. Paape J., and X. Zhao. 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Vet. Res. 39. doi:10.1051/vetres:2007047.

Ibrahim, H. M. M., Y. Y. El-Seedy, and N. A. Gomaa. 2016. Cytokine response and oxidative stress status in dairy cows with acute clinical mastitis. J. Dairy Vet. Anim. Res. 3:1-6.

Jacobsen, S., T. Toelboell, and P.H. Anderson. 2005. Dose dependency and individual variability in selected clinical, haematological and blood biochemical responses after systemic lipopolysaccharide challenge in cattle. Vet. Res. 36:167–178. doi:10.1051/vetres:2004062.

Jensen, K., J. Günther, R. Talbot, W. Petzl, H. Zerbe, H. J. Schuberth, H. M. Seyfert, and E. J. Glass. 2013. Escherichia coli- and Staphylococcus aureus-induced mastitis differentially modulate transcriptional responses in neighbouring uninfected bovine mammary gland quarters. BMC Genomics 14. doi:10.1186/1471-2164-14-36.

Jin, X., K. Wang, H. Liu, F. Hu, F. Zhao, and J. Liu. 2016. Protection of bovine mammary epithelial cells from hydrogen peroxide-induced oxidative cell damage by resveratrol. Oxid. Med. Cell. Longev. 2016:2572175. doi:10.1155/2016/2572175.

Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Mühlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. 167:5887–5894. doi:10.4049/jimmunol.167.10.5887.

Kobayashi, K., C. Kuki, S. Oyama, and H. Kumura. 2016. Pro-inflammatory cytokine TNF-α is a key inhibitory factor for lactose synthesis pathway in lactating mammary epithelial cells. Exp. Cell Res. 340:295-304. doi:10.1016/j.yexcr.2015.10.030.

Kobayashi, K., S. Oyama, T. Uejyo, C. Kuki, M. M. Rahman, and H. Kumura. 2013. Underlying mechanisms involved in the decrease of milk secretion during Escherichia coli endotoxin induced mastitis in lactating mice. Vet. Res. 44. doi:10.1186/1297-9716-44-119.

Komatsu, T., F. Itoh, S. Kushibiki, and K. Hodate. 2005. Changes in gene expression of glucose transporters in lactating and nonlactating cows. J. Anim. Sci. 83:557–564. doi:10.2527/2005.833557x.

Kuhn, N. J., and A. White. 1975. The topography of lactose synthesis. Biochem. J. 148:77–84. doi:10.1042/bj1480077.

Kushibiki, S., H. Shingu, T. Komatsu, F. Itoh, E. Kasuya, H. Aso, and K. Hodate. 2006. Effect of recombinant bovine tumor necrosis factor-α on hormone release in lactating cows. Anim. Sci. J. 77:603–612. doi:10.1111/j.1740-0929.2006.00392.x.

Kvidera, S. K., E. A. Horst, M. Abuajamieh, E. J. Mayorga, M. V. S. Fernandez, and L. H. Baumgard. 2017. Glucose requirements of an activated immune system in lactating Holstein cows. J. Dairy Sci. 100:2360–2374. doi:10.3168/jds.2016-12001.

Lacasse, P., S. Ollier, V. Lollivier, and M. Boutinaud. 2016. New insights into the importance of prolactin in dairy ruminants. J. Dairy Sci. 99:864–874. doi:10.3168/jds.2015-10035.

Lehmann, M., O. Wellnitz, and R. M. Bruckmaier. 2013. Concomitant lipopolysaccharide-induced transfer of blood-derived components including immunoglobulins into milk. J. Dairy Sci. 96:889–896. doi:10.3168/jds.2012-5410.

Lengemann, F. W., and M. Pitzrick. 1986. Effects of endotoxin on mammary secretion of lactating cows. J. Dairy Sci. 69:1250–1258. doi:10.3168/jds.S0022-0302(86)80531-8.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis,

R. Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079. doi:10.1093/bioinformatics/btp352.

Liao, Y., G. K. Smyth, and W. Shi. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30:923–930. doi:10.1093/bioinformatics/btt656.

Lin, Y., X. Sun, X. Hou, B. Qu, X. Gao, and Q. Li. 2016. Effects of glucose on lactose synthesis in mammary epithelial cells from dairy cow. BMC Vet. Res. 12. doi:10.1186/s12917-016-0704-x.

Linzell, J. L. 1967. The effect of very frequent milking and of oxytocin on the yield and composition of milk in fed and fasted goats. J. Physiol. 190:333-346.

Lohuis, J. A. C. M., J. H. M. Verheijden, C. Burvenich, and A. S. J. P. A. M. van Miert. 1988. Pathophysiological effects of endotoxins in ruminants. Vet. Q. 10:117–125. doi:10.1080/01652176.1988.9694158.

Long, E., A. V. Capuco, D. L. Wood, T. Sonstegard, G. Tomita, M. J. Paape, and X. Zhao. 2001. Escherichia coli induces apoptosis and proliferation of mammary cells. Cell Death Differ. 8:808–816. doi:10.1038/sj.cdd.4400878.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15. doi:10.1186/s13059-014-0550-8.

Mačuhová, J., V. Tančin, and R. M. Bruckmaier. 2004. Effects of oxytocin administration on oxytocin release and milk ejection. J. Dairy Sci. 87:1236–1244. doi:10.3168/jds.S0022-0302(04)73274-9.

Masedunskas, A., Y. Chen, R. Stussman, R. Weigert, and I. H. Mather. 2017. Kinetics of milk lipid droplet transport, growth, and secretion revealed by intravital imaging: lipid droplet release is intermittently stimulated by oxytocin. Mol. Biol. Cell 28:935–946. doi:10.1091/mbc.e16-11-0776.

Matsunaga, K., Y. Tsugami, A. Kumai, T. Suzuki, T. Nishimura, and K. Kobayashi. 2018. IL-1β directly inhibits milk lipid production in lactating mammary epithelial cells concurrently with enlargement of cytoplasmic lipid droplets. Exp. Cell Res. 370:365–372. doi:10.1016/j.yexcr.2018.06.038.

McCoard, S. A., A. A. Hayashi, Q. Sciascia, J. Rounce, B. Sinclair, W. C. McNabb, and N. C. Roy. 2016. Mammary transcriptome analysis of lactating dairy cows following administration of bovine growth hormone. Animal 10:2008–2017. doi:10.1017/S1751731116000987.

McFadden, J. W., C. L. Girard, S. Tao, Z. Zhou, J. K. Bernard, M. Duplessis, and H. M. White. 2020. Symposium review: One-carbon metabolism and methyl donor

nutrition in the dairy cow. J. Dairy Sci. 103:5668-5683. doi:10.3168/jds.2019-17319.

Mehrzad, J., C. Desrosiers, K. Lauzon, G. Robitaille, X. Zhao, and P. Lacasse. 2005. Proteases involved in mammary tissue damage during endotoxin-induced mastitis in dairy cows. J. Dairy Sci. 88:211–222. doi:10.3168/jds.S0022-0302(05)72679-5.

Mehrzad, J., H. Dosogne, E. Meyer, and C. Burvenich. 2001. Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. Vet. Res. 32:131–144. doi:10.1051/vetres:2001100.

Merle, R., A. Schröder, and J. Hamann. 2007. Cell function in the bovine mammary gland: a preliminary study on interdependence of healthy and infected udder quarters. J. Dairy Res. 74:174–179. doi:10.1017/S002202990600238X.

Minuti, A., Z. Zhou, D. E. Graugnard, S. L. Rodriguez-Zas, A. R. Palladino, F. C. Cardoso, E. Trevisi, and J. J. Loor. 2015. Acute mammary and liver transcriptome responses after an intramammary Escherichia coli lipopolysaccharide challenge in postpartal dairy cows. Physiol. Rep. 3:e12388. doi:10.14814/phy2.12388.

Mitterhuemer, S., W. Petzl, S. Krebs, D. Mehne, A. Klanner, E. Wolf, H. Zerbe, and H. Blum. 2010. Escherichia coli infection induces distinct local and systemic transcriptome responses in the mammary gland. BMC Genomics. 11. doi:10.1186/1471-2164-11-138.

Moyes, K. M., P. Sørensen, and M. Bionaz. 2016. The impact of intramammary Escherichia coli challenge on liver and mammary transcriptome and cross-talk in dairy cows during early lactation using RNAseq. PLoS One 11:e0157480.

Munford, R. S. 2005. Invited review: Detoxifying endotoxin: Time, place and person. J. Endotoxin Res. 11:69–84. doi:10.1177/09680519050110020201.

Needs, E. C., and M. Anderson. 1984. Lipid composition of milks from cows with experimentally induced mastitis. J. Dairy Res. 51:239–249. doi:10.1017/S0022029900023505.

Nielsen, N. I., T. Larsen, M. Bjerring, and K. L. Ingvartsen. 2005. Quarter health, milking interval, and sampling time during milking affect the concentration of milk constituents. J. Dairy Sci. 88:3186–3200. doi:10.3168/jds.S0022-0302(05)73002-2.

Ontsouka, C. E., R. M. Bruckmaier, and J. W. Blum. 2003. Fractionized milk composition during removal of colostrum and mature milk. J. Dairy Sci. 86:2005–2011. doi:10.3168/jds.S0022-0302(03)73789-8.

Osorio, J. S., J. Lohakare, and M. Bionaz. 2016. Biosynthesis of milk fat, protein, and lactose: Roles of transcriptional and posttranscriptional regulation. Physiol. Genomics 48:231–256. doi:10.1152/physiolgenomics.00016.2015.

Paape, M. J., P. M. Rautiainen, E. M. Lilius, C. E. Malstrom, and T. H. Elsasser. 2002. Development of anti-bovine TNF-α mAb and ELISA for quantitating TNF-α in milk after intramammary injection of endotoxin. J. Dairy Sci. 85:765–773. doi:10.3168/jds.S0022-0302(02)74134-9.

Paixão, M. G., L. R. Abreu, R. Richert, and P. L. Ruegg. 2017. Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis. J. Dairy Sci. 100:7522–7533. doi:10.3168/jds.2017-12547.

Pei, H., L. Li, B. L. Fridley, G. D. Jenkins, K. R. Kalari, W. Lingle, G. Petersen, Z. Lou, and L. Wang. 2009. FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. Cancer Cell 16:259–266. doi:10.1016/j.ccr.2009.07.016.

Persson Waller, K., I. G. Colditz, S. Lun, and K. Östensson. 2003. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. Res. Vet. Sci. 74:31–36. doi:10.1016/S0034-5288(02)00147-9.

Petzl, W., J. Günther, K. Mühlbauer, H.-M. Seyfert, H.-J. Schuberth, J. Hussen, C. Sauter-Louis, A. Hafner-Marx, and H. Zerbe. 2016. Early transcriptional events in the udder and teat after intra-mammary Escherichia coli and Staphylococcus aureus challenge. Innate Immun. 22:294–304. doi:10.1177/1753425916640057.

Petzl, W., J. Günther, T. Pfister, C. Sauter-Louis, L. Goetze, S. von Aulock, A. Hafner-Marx, H.-J. Schuberth, H.-M. Seyfert, and H. Zerbe. 2011. Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun. 18:467–477. doi:10.1177/1753425911422407.

Petzl, W., H. Zerbe, J. Günther, W. Yang, H.-M. Seyfert, G. Nürnberg, and H.-J. Schuberth. 2008. Escherichia coli, but not Staphylococcus aureus triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Vet. Res. 39.

Piskounova, E., M. Agathocleous, M. M. Murphy, Z. Hu, S. E. Huddlestun, Z. Zhao, A. M. Leitch, T. M. Johnson, R. J. DeBerardinis, and S. J. Morrison. 2015. Oxidative stress inhibits distant metastasis by human melanoma cells. Nature 527:186–191. doi:10.1038/nature15726.

Porcheray, F., S. Viaud, A.-C. Rimaniol, C. Léone, B. Samah, N. Dereuddre-Bosquet, D. Dormont, and G. Gras. 2005. Macrophage activation switching: An asset for the resolution of inflammation. Clin. Exp. Immunol. 142:481–489. doi:10.1111/j.1365-2249.2005.02934.x.

Prgomet, C., H. Sarikaya, R. M. Bruckmaier, and M. W. Pfaffl. 2005. Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. J. Vet. Med. Ser. A 52:317–324. doi:10.1111/j.1439-0442.2005.00741.x.

R Core Team. 2018. R: A language and environment for statistical computing. The R Project for Statistical Computing.

Reid, I. M., and R. L. Chandler. 1973. Ultrastructural studies on the bovine mammary gland with particular reference to glycogen distribution. Res. Vet. Sci. 14:334–343. doi:10.1016/S0034-5288(18)33885-2.

Rhoads, R. E., and E. Grudzien-Nogalska. 2007. Translational regulation of milk protein synthesis at secretory activation. J. Mammary Gland Biol. Neoplasia 12:283–292. doi:10.1007/s10911-007-9058-0.

Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zähringer, U. Seydel, and F. Di Padova. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB J. 8:217–225. doi:10.1096/fasebj.8.2.8119492.

Rinaldi, M., R. W. Li, D. D. Bannerman, K. M. Daniels, C. Evock-Clover, M. V. B. Silva, M. J. Paape, B. Van Ryssen, C. Burvenich, and A. V. Capuco. 2010a. A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to E. coli mastitis. Funct. Integr. Genomics 10:21–38. doi:10.1007/s10142-009-0133-z.

Rinaldi, M., R. W. Li, and A. V. Capuco. 2010b. Mastitis associated transcriptomic disruptions in cattle. Vet. Immunol. Immunopathol. 138:267–279. doi:10.1016/j.vetimm.2010.10.005.

Rinaldi, S. F., R. D. Catalano, J. Wade, A. G. Rossi, and J. E. Norman. 2014. Decidual neutrophil infiltration is not required for preterm birth in a mouse model of infection-induced preterm labor. J. Immunol. 192:2315–2325. doi:10.4049/jimmunol.1302891.

Romano, S., Y. Xiao, M. Nakaya, A. D'Angelillo, M. Chang, J. Jin, F. Hausch, M. Masullo, X. Feng, M. F. Romano, and S.-C. Sun. 2015. FKBP51 employs both scaffold and isomerase functions to promote NF-κB activation in melanoma. Nucleic Acids Res. 43:6983–6993. doi:10.1093/nar/gkv615.

Sadler, A. J., F. J. Rossello, L. Yu, J. A. Deane, X. Yuan, D. Wang, A. T. Irving, M. Kaparakis-Liaskos, M. P. Gantier, H. Ying, H. C. H. Yim, E. L. Hartland, A. J. Notini, S. de Boer, S. J. White, A. Mansell, J.-P. Liu, D. N. Watkins, S. Gerondakis, B. R. G. Williams, and D. Xu. 2015. BTB-ZF transcriptional regulator PLZF modifies chromatin to restrain inflammatory signaling programs. Proc. Natl. Acad. Sci. U. S. A. 112:1535–1540. doi:10.1073/pnas.1409728112.

Schmitz, S., M. W. Pfaffl, H. H. D. Meyer, and R. M. Bruckmaier. 2004. Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. Domest. Anim. Endocrinol. 26:111–126. doi:10.1016/j.domaniend.2003.09.003.

Schultze, W. D., and A. J. Bramley. 1982. Effect of Escherichia coli endotoxin-mediated inflammation of one mammary quarter of the bovine udder on diapedesis into other quarters. J. Dairy Res. 49:381–385.

Schwarz, D., U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. 2011. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. J. Dairy Sci. 94:5033–5044. doi:10.3168/jds.2011-4348.

Scott, R. A., D. E. Bauman, and J. H. Clark. 1976. Cellular gluconeogenesis by lactating bovine mammary tissue. J. Dairy Sci. 59:50–56. doi:10.3168/jds.S0022-0302(76)84155-0.

Shamay, A., F. Shapiro, H. Barash, I. Bruckental, and N. Silanikove. 2000. Effect of dexamethasone on milk yield and composition in dairy cows. Ann. Zootech. 49:343–352.

Shamay, A., F. Shapiro, G. Leitner, and N. Silanikove. 2003. Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. J. Dairy Sci. 86:1250–1258. doi:10.3168/jds.S0022-0302(03)73709-6.

Shangraw, E. M., R. O. Rodrigues, M. C. Witzke, R. K. Choudhary, F.-Q. Zhao, and T. B. McFadden. 2020. Intramammary lipopolysaccharide infusion induces local and systemic effects on milk components in lactating bovine mammary glands. J. Dairy Sci. doi:10.3168/jds.2019-18022.

Shapiro, F., and N. Silanikove. 2010. Rapid and accurate determination of D- and L-lactate, lactose and galactose by enzymatic reactions coupled to formation of a fluorochromophore: Applications in food quality control. Food Chem. 119:829–833. doi:10.1016/j.foodchem.2009.07.029.

Shennan, D. B., and C. A. R. Boyd. 2014. The functional and molecular entities underlying amino acid and peptide transport by the mammary gland under different physiological and pathological conditions. J. Mammary Gland Biol. Neoplasia 19:19–33. doi:10.1007/s10911-013-9305-5.

Shuster, D. E., and R. J. Harmon. 1992. High cortisol concentrations and mediation of the hypogalactia during endotoxin-induced mastitis. J. Dairy Sci. 75:739–746. doi:10.3168/jds.S0022-0302(92)77811-4.

Shuster, D. E., R. J. Harmon, J. A. Jackson, and R. W. Hemken. 1991a. Endotoxin mastitis in cows milked four times daily. J. Dairy Sci. 74:1527–1538. doi:10.3168/jds.S0022-0302(91)78313-6.

Shuster, D. E., R. J. Harmon, J. A. Jackson, and R. W. Hemken. 1991b. Reduced lactational performance following intravenous endotoxin administration to dairy cows. J. Dairy Sci., 74:3407–3411. doi:10.3168/jds.S0022-0302(91)78530-5.

Shuster, D. E., R. J. Harmon, J. A. Jackson, and R.W. Hemken. 1991c. Suppression of milk production during endotoxin-induced mastitis. J. Dairy Sci. 74:3763–3774. doi:10.3168/jds.S0022-0302(91)78568-8.

Shuster, D. E., and R. J. Harmon. 1991. Lactating cows become partially refractory to frequent intramammary endotoxin infusions: recovery of milk yield despite a persistently high somatic cell count. Res. Vet. Sci. 51:272–277. doi:10.1016/0034-5288(91)90077-2.

Shuster, D. E., M. E. Kehrli, and M. G. Stevens. 1993. Cytokine production during endotoxin-induced mastitis in lactating dairy cows. Am. J. Vet. Res. 54:80-85.

Silanikove, N., A. Rauch-Cohen, F. Shapiro, S. Blum, A. Arieli, and G. Leitner. 2011. Lipopolysaccharide challenge of the mammary gland in bovine induced a transient glandular shift to anaerobic metabolism. J. Dairy Sci. 94:4468–4475. doi:10.3168/jds.2010-4092.

Silanikove, N., F. Shapiro, U. Merin, Y. Lavon, S. E. Blum, and G. Leitner. 2016. Reduced use of glucose by normoxic cow's mammary gland under acute inflammation: an example of homeostatic aerobic glycolysis. RSC Adv. 6:114644–114657. doi:10.1039/C6RA22934D.

Singh, K., K. M. Swanson, H. V. Henderson, R. A. Erdman, and K. Stelwagen. 2015. The effect of milking reinitiation following extended nonmilking periods on lactation in primiparous dairy cows. J. Dairy Sci. 98:7666–7674. doi:10.3168/jds.2014-8883.

Sordillo, L. M., and S. L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128:104–109. doi:10.1016/j.vetimm.2008.10.305.

Spek, J. W., J. Dijkstra, and A. Bannink. 2016. Influence of milk urea concentration on fractional urea disappearance rate from milk to blood plasma in dairy cows. J. Dairy Sci. 99:3880–3888. doi:10.3168/jds.2015-9421.

Stelwagen, K., S. R. Davis, V. C. Farr, and S. J. Eichler. 1994. Effect of once daily milking and concurrent somatotropin on mammary tight junction permeability and yield of cows. J. Dairy Sci. 77:2994-3001. doi:10.3168/jds.S0022-0302(94)77240-4.

Stelwagen, K., V. C. Farr, and H. A. McFadden. 1999. Alteration of the sodium to potassium ration in milk and the effect on milk secretion in goats. J. Dairy Sci. 82:52-59. doi:10.3168/jds.S0022-0302(99)75208-2.

Stelwagen, K., V. C. Farr, H. A. McFadden, C. G. Prosser, and S. R. Davis. 1997. Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. Am. J. Physiol. Integr. Comp. Physiol. 273:R379–R386. doi:10.1152/ajpregu.1997.273.1.R379.

Stelwagen, K., and C. H. Knight. 1997. Effect of unilateral once or twice daily milking of cows on milk yield and udder characteristics in early and late lactation. J. Dairy Res. 64:487–494. doi:10.1017/S0022029997002458.

Stelwagen, K., D. C. van Espen, G. A. Verkerk, H. A. McFadden, and V. C. Farr. 1998. Elevated plasma cortisol reduces permeability of mammary tight junctions in the lactating bovine mammary epithelium. J Endocrinol 159:173–179.

Stinnakre, M. G., J. L. Vilotte, S. Soulier, and J. C. Mercier. 1994. Creation and phenotypic analysis of alpha-lactalbumin-deficient mice. Proc. Natl. Acad. Sci. 91:6544–6548. doi:10.1073/pnas.91.14.6544.

Sung, M.-H., N. Li, Q. Lao, R. A. Gottschalk, G. L. Hager, and I. D. C. Fraser. 2014. Switching of the relative dominance between feedback mechanisms in lipopolysaccharide-induced NF-κB signaling. Sci. Signal. 7:ra6. doi:10.1126/scisignal.2004764.

Suojala, L., T. Orro, H. Järvinen, J. Saatsi, and S. Pyörälä. 2008. Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows. Acta Vet. Scand. 50. doi:10.1186/1751-0147-50-18.

Thomas, E., N. Zeps, P. Rigby, and P. Hartmann. 2011. Reactive oxygen species initiate luminal but not basal cell death in cultured human mammary alveolar structures: a potential regulator of involution. Cell Death Dis. 2:e189–e189. doi:10.1038/cddis.2011.69.

Tisoncik, J. R., M. J. Korth, C. P. Simmons, J. Farrar, T. R. Martin, and M. G. Katze. 2012. Into the eye of the cytokine storm. Microbiol. Mol. Biol. Rev. 76:16–32. doi:10.1128/MMBR.05015-11.

Turner, C. W. 1934. The functional individuality of the mammary glands of the udder of the dairy cow. Columbia, MO.

Van Den Berg, C., A. J. De Neeling, C. S. Schot, W. N. M. Hustinx, J. Wemer, and D. J. De Wildt. 1992. Delayed antibiotic-induced lysis of Escherichia coli in vitro is correlated with enhancement of LPS release. Scand. J. Infect. Dis. 24:619–627. doi:10.3109/00365549209054648.

van der Kolk, J. H. 1990. The bovine pituitary-adrenocortical axis and milk yield. Vet. Q. 12:114–120. doi:10.1080/01652176.1990.9694253.

van der Kolk, J. H., H. J. Breukink, T. Wensing, and J. A. Mol. 1991. Adrenocortical

function testing in dairy cows and its effect on milk yield. Vet. Q. 13:144–147. doi:10.1080/01652176.1991.9694299.

Vernay, M. C. M. B., O. Wellnitz, L. Kreipe, H. A. van Dorland, and R. M. Bruckmaier. 2012. Local and systemic response to intramammary lipopolysaccharide challenge during long-term manipulated plasma glucose and insulin concentrations in dairy cows. J. Dairy Sci. 95:2540–2549. doi:10.3168/jds.2011-5188.

Verstrepen, L., T. Bekaert, T.-L. Chau, J. Tavernier, A. Chariot, and R. Beyaert. 2008. TLR-4, IL-1R and TNF-R signaling to NF-κB: variations on a common theme. Cell. Mol. Life Sci. 65:2964–2978. doi:10.1007/s00018-008-8064-8.

Waldron, M. R., A. E. Kulick, A. W. Bell, and T. R. Overton. 2006. Acute experimental mastitis is not causal toward the development of energy-related metabolic disorders in early postpartum dairy cows. J. Dairy Sci. 89:596–610. doi:10.3168/jds.S0022-0302(06)72123-3.

Waldron, M. R., T. Nishida, B. J. Nonnecke, and T. R. Overton. 2003. Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. J. Dairy Sci. 86:3447–3459. doi:10.3168/jds.S0022-0302(03)73949-6.

Wall, E. H., and T. B. McFadden. 2007. The milk yield response to frequent milking in early lactation of dairy cows is locally regulated. J. Dairy Sci. 90:716–720. doi:10.3168/jds.S0022-0302(07)71555-2.

Wall, S. K., L. E. Hernández-Castellano, A. Ahmadpour, R. M. Bruckmaier, and O. Wellnitz. 2016. Differential glucocorticoid-induced closure of the blood-milk barrier during lipopolysaccharide- and lipoteichoic acid-induced mastitis in dairy cows. J. Dairy Sci. 99:7544–7553. doi:10.3168/jds.2016-11093.

Wellnitz, O., C. Zbinden, J. Lüttgenau, H. Bollwein, and R. M. Bruckmaier. 2015. Different chronological patterns of appearance of blood derived milk components during mastitis indicate different mechanisms of transfer from blood into milk. J. Dairy Res. 82:322–327. doi:10.1017/S0022029915000345.

Wieman, H. L., J. A. Wofford, and J. C. Rathmell. 2007. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol. Biol. Cell 18:1437–1446. doi:10.1091/mbc.e06-07-0593.

Xi, X., L.-Y. Kwok, Y. Wang, C. Ma, Z. Mi, and H. Zhang. 2017. Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry MSE-based untargeted milk metabolomics in dairy cows with subclinical or clinical mastitis. J. Dairy Sci. 100:4884–4896. doi:10.3168/jds.2016-11939.

Zhao, F.-Q. 2014. Biology of glucose transport in the mammary gland. J. Mammary Gland Biol. Neoplasia 19:3–17. doi:10.1007/s10911-013-9310-8.

Zheng, J., A. D. Watson, and D. E. Kerr. 2006. Genome-wide expression analysis of lipopolysaccharide-induced mastitis in a mouse model. Infect. Immun. 74:1907–1915. doi:10.1128/IAI.74.3.1907-1915.2006.

Ziv, G., I. Hartman, E. Bogin, J. Abidar, and A. Saran. 1976. Endotoxin in blood and milk and enzymes in the milk of cows during experimental Escherichia coli endotoxin mastitis. Theriogenology 6:343–352. doi:10.1016/0093-691X(76)90026-1.