ABSTRACT: Mastitis is a highly prevalent and costly disease of dairy cows that is commonly caused by intramammary bacterial infection. The innate immune response to bacterial penetration of the mammary gland is evoked within hours of infection, and the rapidity and magnitude of this response have been demonstrated to influence the resolution of this disease. Cytokines and other mediators of inflammation are known to play critical roles in the innate immune response to intramammary infection. The objectives of this review are to summarize the current understanding of the cytokine response to intramammary infection, highlight recent findings identifying differences in the cytokine response to various bacterial pathogens, and discuss future research directions that will increase our knowledge of the role of inflammatory mediators in predicting and governing the outcome of mastitis.

Key words: cytokine, dairy cow, inflammation, innate immunity, mastitis

INTRODUCTION

Mastitis is a highly prevalent disease in dairy cows and one of the most costly to the dairy industry (Wells et al., 1998; Wellenberg et al., 2002; Seegers et al., 2003). Mastitis is an inflammatory disease that most commonly results from bacterial infection of the mammary gland. A broad spectrum of bacteria can successfully establish an infection within the mammary gland, including gram-positive, gram-negative, and wall-less (e.g., *Mycoplasma bovis*) bacteria (Wilson et al., 1997; Barkema et al., 1998; Fox et al., 2005). *Staphylococcus aureus*, coagulase-negative staphylococci, and *Streptococcus uberis* are among the most prevalent gram-positive bacteria to cause this disease. Among the gram-negative bacteria that cause mastitis, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Enterobacter* spp. are the most common. The successful establishment and persistence of an intramammary infection are mediated by both intrinsic virulence factors of the bacterial pathogen (Hornef et al., 2002) and the rapidity and nature of the immune response of the cow to the pathogen (Burvenich et al., 2003). In contrast to the adaptive immune system, which requires several days to become capable of exerting a protective response to infection, the innate immune system is poised to respond immediately to the earliest stages of infection and recognize pathogens that have not been encountered previously (Uthaisangsook et al., 2002). Thus, the innate immune system represents the first line of active defense against invading pathogens once they have penetrated the physical barrier of the streak canal. Because the clearance of bacterial pathogens from the gland is often governed by responses that occur in the immediate hours and days after initial infection (Kremer et al., 1993; Kehrli and Shuster, 1994; Lee et al., 2003b), the innate arm of the immune system represents the primary host determinant for dictating the outcome of intramammary infection. Although elements of the innate immune system represent ancient and highly conserved mechanisms by which the host defends itself against pathogens, profound differences in innate immune responses to intramammary pathogens have been identified. This review highlights pathogen-dependent variations in the generation of soluble inflammatory mediators by the host during the innate immune response to intramammary infection and discusses the potential ramifications of these differences.

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CYTOKINE MEDIATORS OF MAMMARY INFLAMMATION

Cytokines

Cytokines are soluble proteins that affect an array of biological processes, including inflammation and immunity. Almost every mammalian cell type has the capability to produce and respond to cytokines (Dinarello, 2007). Although the majority of cytokines are secreted, some are restricted to the cellular membrane. Most cytokines have more than one function and often have redundant effects with other cytokines (Taniguchi, 1995). Because of the high affinity of their receptors, cytokines are highly potent and can elicit biological responses when present in femtomolar to nanomolar concentrations (Wahl et al., 1988; Hall et al., 1989). One of the primary mechanisms by which they exert their effects is through the activation of intracellular signal transduction pathways and the corresponding upregulation of gene expression (Gouwy et al., 2005). Although cytokines play an essential role in the host response to infection, they can have deleterious effects on the host. Thus, there is a fine balance between the positive and negative effects of cytokines on the host that is dictated by the duration, amount, and location of their expression.

Interferon-γ

Interferon (IFN)-γ plays a role in linking the innate and adaptive arms of the immune system and is critical for host immunity against intracellular pathogens. Cellular sources of IFN-γ include lymphocytes, natural killer cells, and cells of monocytic lineage (Schroder et al., 2004; Schoenborn and Wilson, 2007). The influence of IFN-γ on the innate immune system is most evident from its effects on macrophages and neutrophils. Interferon-γ enhances the microbicidal activity of these cells by increasing receptor-mediated phagocytosis, inducing respiratory burst activity, and priming nitric oxide production (Ellis and Beaman, 2004; Schroder et al., 2004). Interferon-γ also upregulates cell-surface major histocompatibility complex (MHC) class I molecule expression, thus promoting the induction of cell-mediated immunity by increasing the likelihood of cytotoxic T-cell recognition of presented foreign peptides (Schroder et al., 2004). Further, IFN-γ upregulates the MHC class II antigen presentation pathway and corresponding CD4+ T-cell activation.

At the transcript level, increases in IFN-γ mRNA have been detected in cells isolated from the milk of mammary glands infected with E. coli (Lee et al., 2006) and S. aureus (Riollet et al., 2001). During the course of naturally occurring mastitis (Hisaeda et al., 2001), as well as in the setting of experimental mastitis induced by E. coli, M. bovis, S. aureus, P. aeruginosa, S. marcescens, and S. uberis (Figure 1A; Bannerman et al., 2004a,b,c, 2005; Kauf et al., 2007a), increases in milk protein concentrations of IFN-γ have also been detected. Concentrations of 20 ng/mL have been detected in cases of naturally acquired mastitis, whereas concentrations ranging from 0.2 to 5 ng/mL have been reported during experimentally induced infections. Interestingly, the greatest concentrations of IFN-γ have been detected in intramammary infections characterized by persistent infection with increased numbers of recoverable bacteria (Bannerman et al., 2004a; Kauf et al., 2007a). This may reflect an attempt by the host to heighten cell-mediated immune responses to eradicate pathogens that are not readily eliminated by earlier activated host innate immune defense mechanisms.

IL-1

Interleukin-1 is a proinflammatory cytokine and one of the most potent endogenous inducers of fever (Dinarello, 1998). This cytokine plays a critical role in the host defense against infection, but dysregulation of its expression can have deleterious consequences to the host. Many of the biological effects of IL-1 on host innate immune responses to infection are similar to those of tumor necrosis factor-α (TNF-α), including activation of endothelial cells and leukocytes, and systemic induction of fever and acute phase protein synthesis (Pruitt et al., 1995; Dinarello, 1996). Correspondingly, the deleterious effects of IL-1 overlap those of TNF-α and include the ability to induce shock, vascular leakage, and multiorgan failure. A variety of cells have been identified as sources of IL-1, including monocytes, macrophages, dendritic cells, lymphocytes, endothelial and epithelial cells, and fibroblasts (Barksby et al., 2007). The expression of IL-1 is induced in response to bacterial, viral, fungal, and parasitic infections, as well as nonmicrobial sources, including TNF-α, IL-12, and complement component 5a (C5a; Goodman et al., 1982; Dinarello, 1996). In addition to being upregulated by cytokines, IL-1 induces the production of such cytokines as IL-1 itself, TNF-α, IL-6, IL-8, and IL-12. In the cases of TNF-α and IL-6, IL-1 can act synergistically to enhance their effects.

Interleukin-1 is expressed as either IL-1α or IL-1β, 2 structurally and functionally similar polypeptides (Pruitt et al., 1995; Dinarello, 1996). Because of differential expression of leader peptide sequences and proteolytic processing, IL-1α generally remains intracellular, whereas IL-1β is generally secreted. Thus, it is postulated that IL-1α, predominantly serves to regulate intracellular events and mediate local inflammation, whereas IL-1β mediates both local and systemic inflammatory responses. Increases in IL-1 expression during bovine mastitis have been characterized most frequently in the setting of experimental E. coli intramammary infection. Several reports, which have used different strains of E. coli, have reported increases in milk concentrations of IL-1 within 18 h of infection (Shuster et al., 1995, 1996, 1997; Riollet et al., 2000; Bannerman et al., 2004c).
The earliest studies used a bioassay to measure IL-1, and thus could not discriminate between IL-1α and IL-1β production (Shuster et al., 1995, 1996, 1997). It has been reported that IL-1α and IL-1β are upregulated at the mRNA level in cells isolated from cows with mastitis (Riollet et al., 2001; Peli et al., 2003) and in bovine mammary epithelial cells exposed to *E. coli* or *E. coli*-derived products in vitro (McClenahan et al., 2005; Pareek et al., 2005; Strandberg et al., 2005; Lahouassa et al., 2007). Although IL-1α is usually not secreted, it can be released from injured or dead cells (Dinarello, 1996). Thus, it is possible that the earlier studies that used a bioassay to measure IL-1 in milk were detecting an increase in both forms.

More recent studies have used antibodies to specifically measure IL-1β (Riollet et al., 2000; Bannerman et al., 2004a,b,c, 2005; Kauf et al., 2007a). Compared with other cytokines measured, the IL-1β response is highly variable, as reflected by the large SE observed in these studies. Maximum milk concentrations of IL-1β after *E. coli* intramammary infection have been reported to range from approximately 0.3 to 8 ng/mL and are consistent with those detected in response to intramammary infusion of bacterial lipopolysaccharide (LPS; Persson Waller et al., 2003), a highly proinflammatory molecule that can induce clinical mastitis resembling that elicited by *E. coli* infection. Other gram-negative bacteria, including *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens*, have been demonstrated to induce a temporal IL-1β response of similar magnitude to that evoked by intramammary infection with *E. coli* (Figure 1B; Bannerman et al., 2004a,b, 2005).

![Figure 1](image-url)
The induction of IL-1β is delayed after intramammary infection with gram-positive or wall-less bacteria compared with infection with gram-negative bacteria (Rambeaud et al., 2003; Bannerman et al., 2004a; Kauf et al., 2007a); however, the magnitude of the IL-1β response is comparable. In studies using different strains of *S. uberis*, milk IL-1β concentrations have been shown to reach 2 to 3 ng/mL at ≥60 h after intramammary infection (Rambeaud et al., 2003; Bannerman et al., 2004a). In quarters infected with *M. bovis*, concentrations approaching 0.5 ng/mL have been detected (Kauf et al., 2007a). There have been conflicting findings concerning whether *S. aureus* can elicit an IL-1β response, because one study detected IL-1β in the milk of infected quarters and another did not (Riollet et al., 2000; Bannerman et al., 2004c). At the mRNA level, IL-1β transcription has been determined to be upregulated in milk cells isolated from quarters chronically infected with *S. aureus* (Riollet et al., 2001).

**IL-6**

Interleukin-6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties. This cytokine is expressed by a variety of cells, including lymphocytes, monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, and fibroblasts, and its expression is induced by bacteria and viruses, as well as by cytokines, such as TNF-α and IL-1β (Biffl et al., 1996; van der Poll and van Deventer, 1998). Interleukin-6 is involved in modulating aspects of both innate and adaptive immunity via its ability to induce fever, B-cell differentiation and corresponding immunoglobulin production, T-cell activation, and enhanced proinflammatory responses of neutrophils (Biffl et al., 1996; Keller et al., 1996). Among the clearly defined anti-inflammatory properties of IL-6 is its ability to inhibit expression of IL-1β and TNF-α, and to stimulate expression of IL-1-receptor antagonist and soluble TNF receptor. Interleukin-6 is a prominent inducer of hepatic synthesis of acute phase proteins, many of which facilitate host clearance of infectious pathogens, the downregulation of the inflammatory response, and the restoration of physiological homeostasis (Moshage, 1997; Papanicolaou et al., 1998). Although TNF-α and IL-1β also induce hepatic acute phase protein synthesis, there is evidence that IL-6 induces the upregulation of a differential set of acute phase proteins (Moshage, 1997). In humans, circulating concentrations of IL-6 are highly correlated with increased mortality, a finding that has been attributed to its ability to persist in the circulation for a longer period of time than other proinflammatory cytokines (van der Poll and van Deventer, 1998; Song and Kellum, 2005). Relative to milk cells isolated from uninfected glands, IL-6 mRNA transcription has been demonstrated to be greater in cells isolated from cows with naturally acquired (Taylor et al., 1997; Riollet et al., 2001) or experimentally induced mastitis (Alituwimi et al., 2003; Lee et al., 2006). These increases in transcript abundance have been detected in cows infected with *E. coli*, as well as with gram-positive bacteria, including *S. aureus* and undefined species of *Streptococcus*. Increased concentrations of the IL-6 protein have been detected in the milk and blood of cows with naturally acquired (Nakajima et al., 1997; Hagiwara et al., 2001; Ohtsuka et al., 2001) and experimentally induced mastitis (Shuster et al., 1997; Dernfalk et al., 2007), as well as those infused with bacterial LPS (Shuster et al., 1993). In the controlled setting of an experimentally induced *E. coli* intramammary infection, increases in milk IL-6 have been observed within 16 h of infection and approximately 1 to 2 h after initial increases in TNF-α and IL-1β (Shuster et al., 1997). Milk and blood concentrations of IL-6 ranging from 10 to 50 ng/mL and from 30 to 90 ng/mL, respectively, have been reported in cases of naturally acquired (Hagiwara et al., 2001) and experimentally induced mastitis (Dernfalk et al., 2007). Similar to transcription at the mRNA level, comparable increases in IL-6 protein have been detected among cows with intramammary infections caused by gram-positive or gram-negative bacteria (Hagiwara et al., 2001). In contrast to several studies establishing a correlation between IL-6 and disease severity in bacterial-mediated human diseases (Brouckaert and Fiers, 1996; van der Poll and van Deventer, 1998; Song and Kellum, 2005), there are conflicting reports among the limited number of studies evaluating the association between severity and outcome of bovine mastitis with blood IL-6 concentrations. Among the findings from 3 independent studies that measured IL-6 concentrations in cows with naturally acquired mastitis, one reported greater blood IL-6 concentrations in cows that were eventually killed because of disease severity compared with cows that recovered (Hagiwara et al., 2001). In contrast, another study reported that cows that survived clinical mastitis had greater blood IL-6 concentrations than those that did not survive (Nakajima et al., 1997). A third study found no difference in blood IL-6 concentrations between cows with mild vs. severe mastitis (Ohtsuka et al., 2001). Thus, there remains a lack of consensus on the relationship between circulating IL-6 concentrations and disease severity in cows with mastitis.

**IL-8**

Interleukin-8 is chemotactic cytokine (i.e., chemokine) that is upregulated in response to infection. In contrast to other well-described chemoattractants, such as C5a, platelet-activating factor, and leukotriene B4, which can attract a variety of leukocytes, IL-8 preferentially recruits neutrophils, and to a lesser extent T lymphocytes (Harada et al., 1994; Kobayashi, 2008). In contrast to the more transient effects of other chemoattractants, IL-8 is able to exert a longer lasting effect, presumably because of its resistance to proteolytic degradation and slower clearance from tissues (Baggiolini and Clark-Lewis, 1992; Hebert and Baker, 1993). Inter-
leukin-8 is produced by an array of cell types, including cells of monocytic lineage, endothelial and epithelial cells, fibroblasts, neutrophils, and T-lymphocytes, and can correspondingly be generated in any tissue (Matsukawa et al., 2000). Its expression is induced by both exogenous (e.g., bacteria, viruses, fungi, parasites, and products derived from these pathogens) and endogenous (e.g., TNF-α and IL-1β) proinflammatory stimuli (Matsukawa et al., 2000; Mukaida, 2003). In addition to its role in attracting neutrophils to sites of infection, IL-8 can activate or augment neutrophil respiratory burst activity, degranulation, and generation of arachidonate metabolites (Mukaida et al., 1998). Thus, IL-8 both recruits and enhances the functioning of neutrophils.

Several studies have confirmed that IL-8 is increased during E. coli mastitis compared with healthy glands. In experimental challenge studies using the well-characterized P4 strain (Bramley, 1976), increases in IL-8 in milk have been detected within 16 h of infection and have been reported to achieve concentrations ranging from 200 to 1,000 pg/mL (Riollet et al., 2000; Lee et al., 2003b; Bannerman et al., 2004c; Vangroenweghe et al., 2004, 2005). In similar studies using a different strain of E. coli, milk IL-8 has been shown to increase within 18 to 24 h of infection and to reach concentrations ranging from 100 to 250 pg/mL (Shuster et al., 1996, 1997). The milk concentrations detected during the course of E. coli intramammary infection are comparable with those detected in response to intramammary infusion of LPS (Bannerman et al., 2003; Lee et al., 2003a). Increases in IL-8 mRNA transcription have also been confirmed in milk cells isolated from E. coli-infected glands (Lee et al., 2006).

Similar to E. coli, other gram-negative bacteria, including K. pneumonia, P. aeruginosa, and S. marcescens, have been shown to induce increases in milk IL-8 concentrations within 20 h of intramammary infection (Figure 1C; Bannerman et al., 2004a,b, 2005). Maximally detected concentrations of approximately 100 pg/mL in response to S. marcescens and P. aeruginosa and approximately 700 ng/mL in response to K. pneumoniae, the latter of which induced severe clinical mastitis, are all within the concentration range reported after E. coli intramammary infection. In contrast to gram-negative bacteria, intramammary infections with gram-positive or wall-less bacteria have been shown to induce a delayed or diminished IL-8 response. In 2 studies using different strains of S. uberis, increases in milk IL-8 were not detected until 30 and 66 h after infection (Rambeaud et al., 2003; Bannerman et al., 2004a), both of which were later time points than those reported for initial increases in IL-8 after intramammary infection with any gram-negative bacteria. Further, maximal concentrations of approximately 35 and 60 pg/mL detected in S. uberis-infected quarters were less than those reported in quarters infected with gram-negative bacteria. Mycoplasma bovis has been shown to evoke increases in milk IL-8 concentrations that are comparable with those elicited by gram-negative pathogens; however, initial increases were not detected until 5 d after infection. In contrast to other gram-positive and gram-negative bacteria, studies using different strains of S. aureus have been unable to detect increases in milk IL-8 after experimental intramammary infection (Riollet et al., 2000; Bannerman et al., 2004c). The differential transcription of IL-8 mRNA has also been confirmed in mammary tissue isolated from cows infected with other strains of E. coli and S. aureus (Yang et al., 2008). However, 2 other studies reported detectable increases in IL-8 transcripts in milk cells isolated from S. aureus-infected quarters (Lee et al., 2006; Tao and Mallard, 2007). In the study by Lee et al., IL-8 mRNA transcription was determined to be less in milk cells isolated from S. aureus than from E. coli-infected quarters. Thus, several independent studies using various strains of E. coli and S. aureus have reported greatly diminished or a complete absence of IL-8 expression in response to intramammary infection by S. aureus.

IL-10

Interleukin-10 plays a central role in limiting inflammation and influencing the nature of the adaptive immune response to infection. It is produced by various cell types, including type 2 helper T lymphocytes (T\(\text{H}2\)), B cells, eosinophils, mast cells, and cells of monocytic lineage, the latter of which are considered to be the major in vivo source of the cytokine (Asadullah et al., 2003). Interleukin-10 exerts a broad antiinflammatory effect on monocytes, macrophages, and neutrophils by inhibiting their production of proinflammatory cytokines, chemokines, and eicosanoids (Moore et al., 2001). Interleukin-10 also induces the upregulation of IL-1 receptor antagonist and soluble TNF receptors, which impair the ability of the proinflammatory cytokines IL-1 and TNF-α, respectively, to exert their effects. In terms of its influence on adaptive immunity, IL-10 impairs the ability of monocytes and macrophages to present antigen to T cells by downregulating MHC class II expression. Further, this cytokine is involved in altering the type 1 helper T lymphocyte (T\(\text{H}1\))/T\(\text{H}2\) balance by suppressing the production of IFN-γ and IL-12, which are involved in promoting a T\(\text{H}1\)-type response (Moore et al., 2001; Conti et al., 2003; Mocellin et al., 2004). By virtue of its ability to stimulate B cell function while impairing T\(\text{H}1\)-type responses, IL-10 shifts the nature of the adaptive immune response to one that is humorally mediated.

Intramammary infections by diverse bacterial pathogens, including E. coli, K. pneumoniae, P. aeruginosa, S. marcescens, S. uberis, and M. bovis, have been shown to evoke an increase in milk concentrations of IL-10 (Bannerman et al., 2004a,b,c, 2005; Kauf et al., 2007a). In contrast, detectable increases in IL-10 expression are not evident in response to S. aureus intramammary infection (Bannerman et al., 2004c). During those infections in which an IL-10 response was observed, initial production of IL-10 was preceded by increases in milk
TNF-α concentrations. In S. aureus-infected quarters, the lack of an IL-10 response corresponded with the lack of induction of TNF-α. These cumulative findings of a relationship between TNF-α production and subsequent IL-10 expression are consistent with experimental studies of inflammatory responses in other species and are presumably due to the ability of TNF-α to stimulate IL-10 production (Wanidworanun and Strober, 1993; Oberholzer et al., 2002). In contrast to other cytokines (e.g., IL-8 and TNF-α), there does not appear to be a clear association between the magnitude of the IL-10 response and the bacterial cell wall type (i.e., gram-positive vs. gram-negative vs. wall-less). For instance, S. uberis has been shown to evoke milk IL-10 concentrations similar to those of E. coli, and M. bovis induces concentrations similar to those observed during intramammary infection by K. pneumoniae or P. aeruginosa (Figure 1D). However, initial and maximal increases in IL-10 production are consistently detected earlier in response to gram-negative bacteria than to gram-positive or wall-less bacteria, and these are similar to findings with TNF-α. Interestingly, in cows with the greatest persistent concentrations of bacteria (i.e., S. aureus, S. uberis, M. bovis) in milk, induction of IL-10 is absent or delayed (Figure 1D; Bannerman et al., 2004a,c; Kauf et al., 2007a). This may indicate that earlier induction of IL-10 is beneficial to the ability of the cow to limit bacterial growth and eradicate the pathogen.

**IL-12**

Similar to IFN-γ, IL-12 serves to bridge the innate and adaptive arms of the immune system, and plays an essential role in modulating the host immune response to bacterial and parasitic intracellular pathogens. The active form of IL-12 is a heterodimeric protein composed of both a p35 and a p40 subunit (Trinchieri, 1998b). The mRNA encoding the p35 subunit has been detected in an array of cell types, whereas the detection of the p40 transcripts has been restricted to cells that produce biologically active IL-12. Monocytes and dendritic cells are believed to be the major sources of IL-12 (Langrish et al., 2004). Neutrophils produce IL-12 to a lesser extent than monocytes; however, their presence in such large numbers at sites of infection presumably renders them a pathophysiologically relevant source of this cytokine (Trinchieri, 1998b). Interleukin-12 production is induced by parasites, fungi, viruses, and bacteria, as well as by purified bacterial products, including LPS, lipoteichoic acid, and enterotoxins (Trinchieri, 1998a). The biological activity of IL-12 is exerted on effector cells of both the innate and adaptive arms of the immune system. By virtue of its ability to stimulate the production of IFN-γ by T cells and natural killer cells, IL-12 contributes to the activation of macrophages (Gately et al., 1998; Trinchieri, 2003). Interferon-γ can, in turn, induce the production of IL-12 by phagocytes, resulting in a positive feedback loop. In addition to IFN-γ, IL-12 has been demonstrated to upregulate other cytokines, including TNF-α, IL-8, and IL-10 (Gately et al., 1998). Through its ability to heighten formation of cytoplasmic granules, as well as to increase granule contents involved in pathogen killing, IL-12 enhances the cytotoxic activity of cytotoxic T cells and natural killer cells (Trinchieri, 1998b). In terms of adaptive immunity, IL-12 plays a critical role in altering the balance between Th1 and Th2 responses by promoting the differentiation of T cells into IFN-γ producing Th1 cells (Langrish et al., 2004). Interleukin-12 also alters antibody responses by enhancing the production of immunoglobulins involved in both opsonization and the facilitation of cell-mediated responses, while impairing the production of immunoglobulins involved in mediating Th2 humoral immune responses (Gately et al., 1998).

Increases in IL-12 mRNA abundance have been detected in cells isolated from cows experimentally infected with E. coli or S. aureus (Aluwaimi et al., 2003; Lee et al., 2006), as well as in those with naturally derived cases of S. aureus mastitis (Riollet et al., 2001). At the protein level, increases in IL-12 have been detected in the milk of cows with experimentally induced mastitis. These increases have all been detected within 32 h of experimental intramammary infection with E. coli, S. aureus, P. aeruginosa, S. marcescens, and S. uberis (Bannerman et al., 2004a,b,c; Kauf et al., 2007a). Because IL-12 and IFN-γ are known to induce reciprocal expression, the finding that IL-12 is increased in response to such diverse bacteria is consistent with reports that these bacteria all evoke IFN-γ expression.

**Transforming Growth Factor-α**

Transforming growth factor (TGF)-α regulates an array of proliferative responses and has been implicated in mediating wound healing, epithelial growth, angiogenesis, and mammary gland morphogenesis (Derynck, 1992). Further, TGF-α has proinflammatory properties and has been demonstrated to upregulate IL-8 (Subauste and Proud, 2001) and PGE₂ production (Bry, 1993), enhance the effects of TNF-α and IL-1β (Unemori et al., 1994; Subauste and Proud, 2001), and induce the breakdown of the endothelial-epithelial barrier function, resulting in edema formation (Ohmura et al., 1990; Buse et al., 1995). Transforming growth factor-α is also well established as promoting tissue repair, mammary epithelial proliferation, and mammary gland morphogenesis (Derynck, 1992). Thus, in the setting of diseases such as mastitis, in which inflammation-induced injury to the epithelial lining is a common sequela to intramammary infection (Capuco et al., 1986), TGF-α may promote inflammation and contribute to the resolution of its effects.

Transforming growth factor-α is produced by an array of cell types, including epithelial cells, fibroblasts,
neutrophils, macrophages, and eosinophils (Calafat et al., 1997). In contrast to many other cytokines, expression of TGF-α can be detected in the milk of healthy mammary glands (Chockalingam et al., 2005; Bannerman et al., 2006). Increases in its expression in the gland have been reported after intramammary infection by a variety of bacterial pathogens, including E. coli, M. bovis, P. aeruginosa, S. agalactiae, and S. aureus (Figure 2A; Sheffield, 1997; Bannerman et al., 2005, 2006; Chockalingam et al., 2005; Kauf et al., 2007a). Consistent with the ability of TGF-α to disrupt mammary epithelial tight junction formation (Buse et al., 1995) and elicit ascites formation (Ohmura et al., 1990), increases in TGF-α have been highly correlated ($r_s = 0.855$) in experimentally induced mastitis studies with increases in milk concentrations of BSA, an indicator of breakdown of the milk-blood barrier. The expression of TGF-α has been shown to be temporally coincident with the onset of early-stage inflammatory responses, including the expression of other proinflammatory cytokines (e.g., IL-8 and TNF-α; Bannerman et al., 2005; Chockalingam et al., 2005; Kauf et al., 2007a). In contrast to these cytokines, however, TGF-α concentrations are typically sustained for a longer period of time after infection. Even in the setting of S. aureus intramammary infection, where IL-8 and TNF-α expression are not induced, TGF-α expression is upregulated. This may indicate that in the absence of the production of other proinflammatory mediators, TGF-α may play a more critical role in the host response to intramammary infection.

**TGF-β**

Transforming growth factor-β, which is distinct from the epidermal growth factor family member TGF-α, is a cytokine that has well-described effects on cell growth and differentiation. At different stages of development of the mammary gland, TGF-β has been reported to regulate ductal growth and patterning, as well as alveolar functional differentiation (Daniel et al., 2001). The ability of TGF-β to regulate these events is largely attributed to its growth inhibitory effects on epithelial cells and stimulatory effects on fibroblasts and other stromal cells (Kolek et al., 2003; Musters et al., 2004). In addition to effects on mammary gland development, TGF-β is reported to moderate inflammation. Although mainly regarded as a suppressor of immune and inflammatory responses, TGF-β does exert some proinflammatory properties, depending on the location and activation state of the cells that it is stimulating and the presence of other cytokines (Letterio and Roberts, 1998; Ashcroft, 1999). The immunoregulatory effects of TGF-β include 1) inhibiting macrophage cytokine and nitric oxide production and respiratory burst activity; 2) limiting IFN-γ production; 3) increasing IL-1 receptor antagonist expression; and 4) enhancing macrophage clearance of injured parenchymal cells, inflammatory cells, and bacterial debris. Thus, TGF-β is involved in mediating mammary gland physiological and pathological processes that are associated with development and inflammation, respectively. Transforming growth factor-β is expressed by a variety of leukocytes (Letterio and Roberts, 1998) as well as other cell types, including epithelial cells (Kwong et al., 2004; Zarzynska et al., 2005). In situ hybridization has been used to confirm that the 3 known mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3 (McCartney-Francis et al., 1998), are all expressed in the bovine mammary gland (Maier et al., 1991). In bovine milk, however, only TGF-β1 and TGF-β2 are expressed at measurable concentrations (Ginjala and Pakkanen, 1998; Pakkanen, 1998), with the latter being expressed as the predominant form (Jin et al., 1991; Chockalingam et al., 2005). Increases in both isoforms have been detected during the course of intramammary infection by diverse bacterial pathogens, including E. coli, M. bovis, P. aeruginosa, and S. aureus (Figure 2B; Bannerman et al., 2005, 2006; Chockalingam et al., 2005; Kauf et al., 2007a). In response to all these infections, sustained increases in the expression of TGF-β1 and TGF-β2 are not detected until ≥32 h after initial infection. In comparison with the increases in proinflammatory cytokines reported in these studies, the induction of TGF-β is markedly delayed. Based on the inherent pleiotropic properties of this cytokine in regulating mammary gland development and inflammation, it is likely that TGF-β plays a role in moderating the inflammatory response to intramammary infection and the ensuing tissue repair.

**TNF-α**

Tumor necrosis factor-α is a highly proinflammatory cytokine with both beneficial and injurious properties (van der Poll and Lowry, 1995). At the local level, TNF-α promotes endothelial activation and the recruitment of leukocytes to the site of infection, the latter of which are also activated by TNF-α (Brouckaert and Fiers, 1996). The systemic effects of TNF-α include the induction of fever and acute phase protein synthesis. Although these local and systemic effects are beneficial to the host innate immune defense against infection, TNF-α is associated with heightened inflammatory responses that can threaten the life of the host. Specifically, TNF-α has been shown to induce shock, tissue injury, vascular leakage, multiorgan failure, and dysregulated coagulopathy (Tracey and Cerami, 1994; van der Poll and Lowry, 1995). Tumor necrosis factor-α is able to exert its effects directly or indirectly by stimulating the production of secondary mediators. Multiple cell types have been shown to produce TNF-α, including macrophages, lymphocytes, neutrophils, and epithelial cells (Angelini et al., 2005). Inducers of TNF-α production include viral, fungal, and parasitic pathogens, bacterial wall products and toxins, other cytokines such as IL-1 and IFN-γ, and complement components.
Similar to other bacterially mediated diseases, TNF-α is produced during bovine mastitis, and elevated concentrations of this cytokine have been detected in both milk and blood after intramammary infection. Tumor necrosis factor-α is undetectable in healthy quarters; however, experimental intramammary infection with a low inoculum (<100 cfu/quarter) of *E. coli* results in increased TNF-α concentrations within 18 h of infection that range from approximately 3 to 15 ng/mL in milk (Shuster et al., 1995, 1996, 1997; Riollet et al., 2000; Lee et al., 2003b; Bannerman et al., 2004c; Dernfalk et al., 2007). These concentrations are similar to those detected after intramammary infusion of *E. coli*-derived LPS (Rainard and Paape, 1997; Paape et al., 2002; Lee et al., 2003a; Persson Waller et al., 2003). Experimental infection with a greater inoculum (10^3 cfu/quarter) of *E. coli* has been reported to result in the detection of TNF-α concentrations that approach 100 to 200 ng/mL in milk and detectable concentrations of TNF-α in blood between 0.1 to 10 ng/mL (Hirvonen et al., 1999; Blum et al., 2000; Hoeben et al., 2000). In cows with naturally acquired *E. coli* intramammary infections, milk TNF-α concentrations have been shown to range from 100 pg/mL to 100 ng/mL (Hisaeda et al., 2001; Slebodzinski et al., 2002).

Similar to *E. coli*, intramammary infections with other gram-negative bacteria, including *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens*, evoke similar temporal TNF-α responses (Figure 2C). Maximal milk concentrations detected in response to *P. aeruginosa* and *S. marc-

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**Figure 2.** Cytokine and complement responses to intramammary infection with different bacterial pathogens. Milk samples were collected immediately before (time 0) and at various time points after intramammary infusion of saline (dashed line with cross-marked symbols), *Mycoplasma bovis* (dashed line with open diamonds), *Staphylococcus aureus* (dashed line with open circles), *Streptococcus uberis* (dashed line with open squares), *Escherichia coli* (solid line with closed circles), *Klebsiella pneumoniae* (solid line with closed squares), *Pseudomonas aeruginosa* (solid line with closed diamonds), or *Serratia marcescens* (solid line with closed triangles). The concentrations of transforming growth factor-α (TGF-α; A), transforming growth factor-β1 (TGF-β1; B), tumor necrosis factor-α (TNF-α; C), and complement factor 5a (C5a; D) were all determined by ELISA. Results are a compilation of data published previously (Bannerman et al., 2004a,b,c, 2005, 2006; Chockalingam et al., 2005; Kauf et al., 2007a).
Acute Phase Proteins

Produced in cells isolated from quarters infected with S. aureus, TNF-α mRNA transcription is markedly reduced in glands (Riollet et al., 2001; Alluwaimi et al., 2003). The finding of greater TNF-α in cows with more severe mastitis that developed after experimental infection is consistent with reports of a correlation between the concentration of this cytokine and the severity of naturally occurring coliform mastitis (Ohtsuka et al., 2001) and other bacterially mediated diseases (Waage et al., 1987; Girardin et al., 1988).

In contrast to gram-negative bacteria, gram-positive bacteria or wall-less bacteria have been reported to produce detectable milk concentrations of TNF-α at concentrations comparable with infection (Kauf et al., 2007b). In addition to cytokines, changes in the expression of other mediators of inflammation have been characterized in the setting of bovine mastitis. Acute phase proteins produced in response to bacterial wall products and cytokines (e.g., TNF-α, IL-1β, and IL-6) have varying, and in some cases poorly defined, roles in moderating inflammation. Increases in milk and blood concentrations of serum amyloid A (SAA), haptoglobin, and LPS-binding protein (LBP) have been reported in response to naturally acquired and experimentally induced intramammary infections (Hirvonen et al., 1999; Eckersall et al., 2001; Gronlund et al., 2003, 2005; Bannerman et al., 2004c). Based on results presented across studies investigating increases in SAA and LBP in response to infection by various pathogens, the acute phase response is highly conserved. Even in response to S. aureus infection, which is characterized by the absence of production of at least one cytokine known to induce acute phase protein synthesis (i.e., TNF-α), comparable induction of LBP has been observed relative to that seen in cows infected with E. coli (Bannerman et al., 2004c). Comparable maximal circulating SAA concentrations of approximately 200 to 800 µg/mL have been reported in response to intramammary infection with such diverse pathogens as E. coli (Hirvonen et al., 1999), S. aureus (Gronlund et al., 2003), P. aeruginosa (Bannerman et al., 2005), and M. bovis (Kauf et al., 2007a), or after infusion of LPS (Lehtolainen et al., 2004). Thus, despite known differences in the cytokine response evoked by diverse mastitis pathogens, hepatic synthesis of acute phase proteins is highly conserved.

C5a

Complement activation, which is triggered by antibody immune complexes, bacterial cell surface sugars, and LPS, is characterized by a cascading series of proteolytic events leading to the deposition of a pore-forming complex on the surface of bacteria and concomitant bacterial killing (Gasque, 2004). Intermediate steps leading up to the formation of this complex result in the generation of complement-activation products with proinflammatory properties. The generation of one of these products, C5a, has been well characterized in bacterial-mediated diseases of humans, as well as in bovine mastitis. It is among the most potent chemoattractants for neutrophils and also serves as a chemoattractant for cells of monocytic lineage (Guo and Ward, 2005). In addition, C5a enhances the phagocytotic and respiratory burst activities of these cells and induces neutrophil production of other proinflammatory molecules, including leukotrienes and thromboxanes. Complement component 5a also directly activates the vascular endothelium and elicits adhesion molecule and cytokine expression, which can facilitate leukocyte recruitment and activation. Other inflammatory effects associated with C5a include vasodilation and dysregulated coagulation (Ward, 2004).

Various bacteria have been demonstrated to induce complement activation and corresponding generation of C5a during intramammary infection (Figure 2D),

SOLUBLE NONCYTOKINE MEDIATORS
OF MAMMARY INFLAMMATION

Acute Phase Proteins

In addition to cytokines, changes in the expression of other mediators of inflammation have been characterized in the setting of bovine mastitis. Acute phase
Eicosanoids are fatty acid-derived mediators that are involved in regulating an array of inflammatory processes, including vascular tone, leukocyte recruitment and function, edema, fever, and platelet aggregation (Williams and Higgs, 1988; Bottoms and Adams, 1992). Arachidonic acid is the main precursor of such well-characterized eicosanoids as prostaglandins, thromboxanes, and leukotrienes. Although arachidonic acid is found in all mammalian cell membranes, differential expression of enzymes involved in the generation of its various metabolites confers a differential ability of cell types to produce a given eicosanoid (Ball et al., 1986; Janniger and Racis, 1987). The catabolism of arachidonic acid into prostaglandins and thromboxanes is mediated by cyclooxygenase, whereas the generation of leukotrienes is mediated by lipoxygenase (Williams and Higgs, 1988). As mentioned above, the biological effects of eicosanoids are varied and a given eicosanoid can enhance or counteract the effects of another eicosanoid. Thromboxanes (e.g., thromboxane A₂) can induce vasoconstriction and platelet aggregation, whereas certain prostaglandins (e.g., prostacyclin) promote vasodilation and inhibit platelet aggregation (Ball et al., 1986; Feuerstein and Hallenbeck, 1987). Prostaglandin E₂, which is a potent inducer of fever, also induces vasodilation (Williams and Higgs, 1988). Leukotrienes are involved in promoting leukocyte recruitment and enhancing leukocyte respiratory burst activity and cytokine production (Zipser and Laffi, 1985; Williams and Higgs, 1988; Bottoms and Adams, 1992). Concerning leukocyte recruitment, leukotriene B₄ has been reported to be among the most potent endogenous chemoattractants that are known to exist. Together, the eicosanoids are potent mediators of inflammation with wide-ranging effects on the host innate immune responses to infection and injury.

Consistent with their role in mediating inflammatory processes, increases in the concentrations of eicosanoids have been detected during mastitis; however, there are conflicting reports concerning which eicosanoids become elevated. In studies investigating the eicosanoid response to intramammary infusion of LPS, one study reported that milk concentrations of PGF₁α and PGF₂α, but not PGE₂ or thromboxane B₂, increased after infusion of 50 µg of LPS (Giri et al., 1984). In contrast, another study reported that milk concentrations of thromboxane B₂, but not PGF₂α, increased after infusion of 10 µg of LPS (Anderson et al., 1986). Although the absence of changes detected in PGF₂α in the latter study could be attributed to the reduced concentration of LPS that was infused, it remains unclear why infusion of a greater dose of LPS failed to induce a detectable thromboxane B₂ response. In addition to prostaglandins and thromboxanes, there is indirect evidence for a role of leukotrienes in mediating the response to LPS, because intramammary infusion of a leukotriene biosynthesis inhibitor has been reported to diminish LPS-induced mammary gland recruitment of neutrophils (Waller, 1997).

Experimental intramammary infection with E. coli has been shown in a study to result in increases in milk concentrations of prostacyclin, PGE₂, and thromboxane B₂ (Peter et al., 1990), whereas a similar study failed to detect significant increases in thromboxane B₂, which the authors attributed to large biological variability (Anderson et al., 1985). Increases in leukotriene B₄ and PGF₂α have also been detected in milk obtained from cows experimentally infected with another gram-negative pathogen, K. pneumoniae (Zia et al., 1987; Rose et al., 1989); however, Zia et al. (1987) failed to detect significant increases in PGE₂ or thromboxane A₂, which they also ascribed to large animal variability. Gram-positive pathogens can also evoke an eicosanoid response, because increases in PGE₂, PGF₂α, and thromboxane B₂ have been detected in milk samples obtained from naturally occurring cases of mastitis, in which the causative agent was identified as either S. uberis, S. aureus, S. dysgalactiae, or Micrococcus species (Atroshi et al., 1986, 1987). Similarly, in chronic cases of mastitis caused by coagulase-negative staphylococci, S. uberis, S. agalactiae, or S. aureus, increases in leukotriene...
B₃ have been detected in quarters infected with these gram-positive bacteria (Boutet et al., 2003). These results indicate that intramammary infections by diverse mastitis pathogens elicit increased generation of eicosanoids in the mammary gland. The conflicting abilities of different research groups to consistently detect increases in individual eicosanoids during the course of mastitis may limit the usefulness of these inflammatory mediators as biomarkers of disease severity and progression.

**FUTURE DIRECTIONS AND CHALLENGES**

**Identification of Cellular Sources of Pathophysiologically Relevant Concentrations of Cytokines**

The significance of the contribution of individual cell types to the production of cytokines in vivo during the course of mastitis remains unclear. Although it is well known that cells of monocytic and lymphocytic lineages are major sources of cytokines during infection (Gouwy et al., 2005), there is evidence that other cell types may be significant sources of cytokines in the mammary gland and other tissues. Bovine mammary epithelial cells have the capacity to produce cytokines, and in vitro exposure of these cells to bacteria or bacterial wall products has been demonstrated to elicit their expression of IL-1β, IL-6, IL-8, and TNF-α (Bondjellab et al., 1998; Okada et al., 1999; Wellnitz and Kerr, 2004; McClanahan et al., 2005, 2006; Pareek et al., 2005; Strandberg et al., 2005; Lahouassa et al., 2007; Yang et al., 2008). Bovine neutrophils, which can reach concentrations in excess of 5 × 10⁷ cells/mL of milk during mastitis, have been shown to produce IFN-γ, IL-1β, TNF-α, and IL-12 when stimulated in vitro (Sohn et al., 2007). Although these studies suggest a capacity for epithelial cells and neutrophils to contribute to cytokine production in the mammary gland, more definitive studies are needed to elucidate the cellular sources of cytokines in vivo and the relative amounts that different cell types produce. Experiments using laser capture microdissection in combination with quantitative real-time PCR to isolate specific cell types and quantify their expression of inflammatory genes may provide a more accurate understanding of the role of different cells in mediating the innate immune response to intramammary infection.

**Cytokines as Therapeutics and Biomarkers for Dictating and Predicting the Outcome of Intramammary Infections**

In both human (Aoki and Xing, 2004; Villar and Dongari-Bagtzoglou, 2006) and veterinary medicine (Kehrli et al., 1991; Nickerson, 1991), the therapeutic use of recombinant cytokines for the treatment of infections has been explored. In terms of bovine mastitis, granulocyte colony-stimulating factor (Nickerson et al., 1989), granulocyte-macrophage colony-stimulating factor (Daley et al., 1993; Takahashi et al., 2004; Wedlock et al., 2004), IFN-γ (Sordillo and Babiiuk, 1991), IL-1β (Daley et al., 1993), IL-2 (Daley et al., 1993; Erskine et al., 1998), and IL-8 (Takahashi et al., 2005) have been demonstrated to prevent, improve host clearance of, or enhance the efficacy of antimicrobial treatment of intramammary infections. The efficacy of these cytokines has been attributed to their immunomodulatory properties. A recent study, which demonstrated that intramammary infusion of LPS 24 h after S. aureus intramammary infection reduced milk bacterial concentrations during the period in which milk TNF-α concentrations were increased (Kauf et al., 2007b), further supports a role for cytokines in mediating the outcome of mastitis. In the case of S. aureus mastitis, for which 3 independent studies have established the lack of induction of major proinflammatory cytokines (Riollet et al., 2000; Bannerman et al., 2004c; Yang et al., 2008), administration of proinflammatory cytokines may be helpful in the treatment of these infections. However, in response to intramammary infections caused by pathogens such as E. coli, in which a highly proinflammatory state is evoked, and when exacerbated, can threaten the life of the cow, administration of antiinflammatory cytokines may be beneficial (Sordillo and Peel, 1992). Because of the pathogen-dependent differences in the kinetics and magnitude of cytokine responses, cytokine therapy may need to be tailored to individual pathogens to be successful.

In addition to their potential therapeutic application, cytokines may be useful biomarkers of disease severity and outcome. For example, in quarters persistently infected with the greatest numbers of bacterial colony forming units, IFN-γ concentrations have been shown to be either greater or elevated longer than in quarters in which pathogens are readily cleared or are present at reduced numbers (Figure 1A). Further, in our various studies of the innate immune response to different pathogens, the greatest concentrations of milk C5a were detected in cows infected with strains of K. pneumoniae and S. uberis that induced clinical mastitis of such severity that the studies had to be halted prematurely (Figure 2D). In contrast, intramammary infections with other pathogens that induced less severe symptoms of clinical mastitis were accompanied by decreased milk concentrations of C5a. Because the greater concentrations of C5a may be specific for intramammary infections by K. pneumoniae and S. uberis, additional studies are warranted to investigate whether there is a relationship between complement activation and infections caused by different strains of the same species of bacteria that induce differential disease severity. Finally, the absence of IL-8 and TNF-α production in response to intramammary infection appears to be highly specific for S. aureus (Figures 1C and 2C).
and has been confirmed in 3 independent studies, all of which used different strains (Riollet et al., 2000; Bannerman et al., 2004c; Yang et al., 2008).

For cytokines to be useful as biomarkers of disease, there is a need to have standardized reagents for measuring their concentrations in biological samples. For many years, many of the reagents and antibodies necessary for the quantification of bovine cytokines and other mediators of inflammation were not commercially available. In some cases, various laboratories developed ELISA to the same bovine cytokines by using different antibodies and recombinant standards, but there have been no attempts to evaluate whether the results from these ELISA are comparable. Progress is being made, however, because commercial sources (e.g., CytoCen, Utrecht, the Netherlands; Pierce Biotechnology Inc., Rockford, IL; Serotec Ltd., Oxford, UK; Veterinary Medical Research and Development Inc., Pullman, WA) of recombinant bovine cytokine proteins and antibodies that recognize bovine cytokines are becoming increasingly available (Entrican et al., 2009). Further, large-scale efforts to develop and standardize these reagents are being undertaken by a variety of consortia, including the US Veterinary Immune Reagent Network (http://vetimm.org; last accessed Oct. 21, 2008) and the Immunological Toolbox (http://www.immunologicaltoolbox.co.uk/; last accessed Oct. 21, 2008).

Conclusions

The ability of bacteria to establish infection is determined, in part, by the nature and rapidity of the corresponding host innate immune response. Over the past decade, there has been a marked increase in the understanding of the upregulation of cytokines and other soluble mediators of inflammation during the course of intramammary infection. Further studies are warranted to evaluate whether the profiling of cytokine expression can predict disease outcomes and whether identification of differential cytokine expression may have diagnostic application for guiding the treatment of bovine mastitis.

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