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The Innate Immunity in Bovine Mastitis: The Role of Pattern-Recognition Receptors

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ABSTRACT

Mastitis is the most costly disease for dairy farmers and industry, which are mainly caused by the entry of bacteria to the teat canal. Shortly after the entry of the invading bacteria, the innate immunity recognizes the invading pathogen through pattern recognition receptors and initiates the inflammatory response necessary to eliminate the invading bacteria. This initial inflammatory response releases cytokines and chemoattractants for the rapid and massive influx of neutrophils from the blood to the site of infection which form the first line of cellular defense against bacteria This article reviewed the role of the most recent knowledge regarding the innate immunity in bovine mastitis focusing in the two major mastitis pathogens: *Escherichia coli* and *Staphylococcus aureus* the *S. aureus* appears to mostly circumvent the host immune response, as the Toll-Like Receptors (TLRs) signaling pathways. The Intramammary Infections (IMIs) by this bacteria result in a very moderate host response with minimal observable innate immune response, which are related to well-known ability to this pathogen to establish chronic IMI. Otherwise, *E. coli* elicits a strong and earlier response, mainly through TLR4, that is associated with the severity of the mastitis and the clinical manifestation commonly observed in dairy cows infected with this pathogen. Suboptimal and dysfunctional mammary defenses may contribute to the development of severe acute inflammation or chronic mastitis that adversely affects the milk production and quality. Thus, a better understanding of mastitis pathogen interaction to the host may be useful for future control of mastitis.

Keywords: Dairy Cows, Intramammary Infections, Mammary Gland, PAMPs, Toll-Like Receptors

1. INTRODUCTION

Mastitis is the most costly disease for dairy farmers and industry (Hujips *et al*., 2008; Hogeveen *et al*., 2011). Bovine mastitis is defined as an inflammatory condition of the mammary gland in response to injury, which serve to destroy and neutralize infectious agents and promote healing and the return to normal function. More than 130 microorganisms can cause mastitis, although this disease is usually caused by some groups of bacteria (Wellenberg *et al*., 2002; Hillerton and Berry, 2005). In the last few years, antimicrobial resistance has been growing concern worldwide. Thus, in an attempt to reduce the impact of mastitis and decrease the use of antimicrobials on dairy farms, there have been numerous efforts to try to exploit the immune capacity of the bovine mammary gland

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to stimulate the animal's natural defense mechanisms. These facts can reduce the use of antimicrobials and also minimize the development of resistance of bacterial strains (Wellnitz and Bruckmaier, 2012).

The role of Toll-Like Receptors (TLRs) in innate and adaptive immunity has been subject of many good reviews (Medzhitov, 2001; Takeda *et al*., 2003; Akira and Takeda, 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Hornung and Latz, 2010; Takeuchi and Akira, 2010; Kawai and Akira, 2011; Prince *et al*., 2011). Therefore, there is a need to summarize the role of these new concepts in bovine mastitis. In this review, we focus on the most recent knowledge about TLRs in bovine mastitis regarding their role in the major mastitis pathogens: *Escherichia coli* and *Staphylococcus aureus*.

1.1. Innate Immunity

Bovine mastitis is initiated by the entry of bacteria through the teat canal and soon after is characterized by an important inflammatory response. Shortly after entry of the invading pathogen, the resident leukocytes together with epithelial cells initiate the inflammatory response necessary to eliminate the invading bacteria (Paape *et al*., 2003; Rainard and Riollet, 2006; Aitken *et al*., 2011). These cells release chemoattractants for the rapid recruitment of polymorphonuclear neutrophil leukocytes to the site of infection and consequently the Somatic Cell Count (SCC) increases, which represents different cells types present in milk, including leukocytes and epithelial cells (Paape *et al*., 2003; Souza *et al*., 2012). The marked increase in milk SCC during infection is mainly due to influx of neutrophils from blood to the mammary gland, which neutrophils can represent over 90% of leukocyte population in milk from infected udder quarters in contrast to low numbers of this cell population in uninfected ones (Paape *et al*., 2003; Pyorala *et al*., 2003; Souza *et al*., 2012).

Neutrophils are essential for innate host defense against invading microorganisms and eliminate pathogens by a process known as phagocytosis. During phagocytosis, neutrophils produce reactive oxygen species, including superoxide, hydrogen peroxide and hypochlorous acid and release granule compounds into pathogen-containing vacuoles to kill the invading pathogen (Paape *et al*., 2003; Mehrzad *et al*., 2005; Rainard and Riollet, 2006; Prince *et al*., 2011). Thus, the rapid influx of neutrophils with high antimicrobial activity to the foci of infection is the main process that leads to the elimination of infection (Mehrzad *et al*., 2005). This importance was demonstrated by Mehrzad *et al*. (2005) who described that SCC in moderate cows increase faster that Colony Forming Units (CFU) of *E. coli* bacteria, whereas in severe cows the results were reversed.

Conversely, inflammation and tissue injury, as caused by the influx of neutrophils, can result in the release of endogenous TLRs ligands, known as Damage-Associated Molecular Patterns (DAMPs). DAMPs act in an autocrine manner, alerting the host of damage, but can also amplify inflammation leading to further tissue damage (Prince *et al*., 2011). In contrast, an apoptosis differentiation program facilitates the resolution of neutrophil-mediated inflammatio. It has been suggested that phagocytosis initiates molecular cascade of events that accelerates apoptosis of this leukocyte population. Thus, as neutrophils can accumulate rapidly at sites of infection and there is a concomitant potential to cause severe tissue destruction if they undergo necrosis lysis and release cytotoxic granule and reactive oxygen species onto host tissues. Thus, apoptosis can be viewed as the terminal stage of neutrophil-induced inflammation (Kobayashi *et al*., 2003).

Recognition of microbial pathogens is an essential element for initiation of innate immune responses such as inflammation and is mediated by germline-encoded Pattern-Recognition Receptors (PRRs) that recognize molecular structures that are broadly shared by pathogens, known as Pathogen-Associated Molecular Patterns (PAMPs). Upon PAMP recognition, PRRs initiate a serious of signaling programs that execute the first line of host defensive responses necessary for killing infectious microbes (Medzhitov, 2001; Takeda *et al*., 2003; Akira and Takeda, 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeuchi and Akira, 2010; Kawai and Akira, 2011; Prince *et al*., 2011). TLRs were the first PRRs identified. They are also the best characterized PRRs and recognize a wide range of PAMPs. They are expressed either on the cell surface or associated with intracellular vesicles (Medzhitov, 2001; Takeda and Akira, 2004). To date, 10 functional TLRs have been identified in bovine (Menzies and Ingham, 2006). These 10 TLRs and Nucleotide-binding Oligomerization Domain (NOD) 1 and 2 was detected in tissue from alveolar, ductal, gland cistern and teat canal from infected and healthy quarters, with TLR8 having the least expression in comparison to the other PRRs (Whelehan *et al*., 2011). Functional analysis of mammalian TLRs has revealed that they recognize specific patterns of microbial components that are conserved among pathogens (Takeda and Akira, 2004). Each TLR detect distinct PAMPs derived from bacteria, viruses, mycobacteria, fungi and parasites. For instance, these include lipoproteins (recognized by TLR1, TLR2 and TLR6), flagellin (TLR5), lipopolysaccharide (LPS) (TLR4) and a 6-base DNA motif consisting of an unmethylated CpG dinucleotide motifs (CpG DNA) that are rarely found in higher vertebrates (TLR9) (**Table 1**) (Medzhitov, 2001; Takeda *et al*., 2003; Akira and Takeda, 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeuchi and Akira, 2010; Kawai and Akira, 2011).

Table 1. Toll-like receptors and their ligands*

Receptor	Ligand	Origin of ligand
TLR1	Triacyl Lipopeptides	Bacteria and mycobacteria
TLR2	Lipoprotein/Lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermitis*
	Zymosan	Fungi
TLR3	Double-stranded RNA	Viruses
TLR4	Lypopolysaccharide	Gram-negative bacteria
	Fusion protein	Respiratoty syncytial virus
TLR5	Flagelin	Bacteria
TLR6	Diacil lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Single stranded RNA	Viruses
TLR8	Single stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.

Adapted from Akira and Takeda (2004); N.D.: Not Determined; *Only ligands that can be related to mastitis pathogens was included

For instance for the role of TLRs in bovine mammary gland, it was found that LPS induced the expression of the chemokines MCP-1, MCP-2 and MCP-3 and slightly increase in CXCL8. Conversely, peptidoglycan combined with Lipotechoic Acid (LTA) induced the expression of MCP-1 and a slightly increase in MCP-3 expression. Indeed, no significant expression for any of the chemokines was observed when induced by CpG-DNA (Mount *et al*., 2009).

Furthermore, it should note that the TLRs can act together with other molecules or other TLRs. For instance, TLR4 requires other molecules in addition to TLR4 to recognize LPS. LPS binds to the LPS-Binding Protein (LBP) present in serum and this LPS-LBP complex is subsequently recognized by CD14, which is expressed on monocytes/macrophages and neutrophils. Moreover, LPS stimulation is followed by the increased physical proximity between CD14 and TLR4 in the membrane, suggesting that CD14 and TLR4 may interact in LPS signaling. Indeed, the TLR2 act in cooperation at least with two other TLRs: TLR1 and TLR6. So, the formation of heterodimers between TRL2 and either TLR1 or TLR6 dictates the specificity of ligand recognition (Medzhitov, 2001; Takeda *et al*., 2003; Akira and Takeda, 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeuchi and Akira, 2010; Kawai and Akira, 2011). Another factor that can influence the innate immune response in bovine is cell maturation, as demonstrated for the same monocytes subsets-monocytes, macrophages and dentritic cells, which have different responses to the same TLR agonist (Werling *et al*., 2004).

All TLR signal transduction pathways are known to activate NF-κB factors (Akira and Takeda, 2004). MyD88 (myeloid differentiation primary-response protein 88) dependent pathways are associated with early-phase NF-κB response whereas as MyD88 independent pathways are associated with late-phase NF-κB response. These NF-κB factors subsequently enter the nucleus and bind to target promoters. A wealth of pro-inflammatory regulated genes feature NF-κB attachment sites in their promoter region and transcription factor complex act as a main switch to orchestrate immune defense genes against bacterial infection, as production of several pro-inflammatory cytokines.

Thus, the innate immune system uses various PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The principal functions of PRRs include: opsonization, activation of complement and coagulation cascade, phagocytosis, activation and induction of apoptosis (Medzhitov, 2001). For instance, TLR signaling pathway by bacteria regulated phagocytosis at multiple steps including internalization and phagossome maturation (Blander and Medzhitoz, 2004).

The importance of the innate immunity (TLR2, Tumor Necrosis Factor (TNF)-α, interleukin (IL-)1β, IL-6, IL-8 complement factor C3, lactoferrin and RANTES) was also demonstrated by the significantly elevated expression of these innate immune genes in less-susceptible cattle when compared to high susceptibility group by detection of Quantitative Trait Loci (QTL) affecting mastitis (Griesbeck-Zilch *et al*., 2009).

1.2. Mastitis caused by *Escherichia Coli*

E. coli is among the major mastitis pathogens responsible for clinical mastitis in dairy cows, but the infection are normally cleared by the immune system within a few days. Indeed, in last few decades, with the improvement of mastitis control programs, which leads to herds with low SCC, the clinical mastitis has become a major problem in many well-managed dairy herds that successfully controlled contagious pathogens (Green *et al*., 2004). Gram-negative bacteria, such as *E. coli*, are generally regarded as environmental pathogens, however contagious behavior of these pathogens has been proposed (Burvenich *et al*., 2003; Dogan *et al*., 2006; Suojala *et al*., 2011). With this in mind, it has been suggested that clinical bovine *E. coli* mastitis isolates differ from cowshed environmental *E. coli* isolates and may form a subset of environmental *E. coli* population . Mastitis isolates showed faster growth in the udder medium and can evade the host cellular innate immune response (Blum *et al*., 2008).

E. coli express a variety of virulence factors, but no coherence between the severity of disease and specific virulence factors could be defined (Wenz *et al*., 2006; Suojala *et al*., 2011; Schukken *et al*., 2011). The ability to grow in mammary secretions and to liberate LPS is crucial in the pathogenesis of *E. coli* mastitis. The faster bacterial numbers increase in the mammary gland, more LPS is present in the mammary gland and faster inflammatory response and clinical disease may occur (Mehrzad *et al*., 2008). Sensing the pathogen and initiating an immune response depends on the initial number of bacteria present at the start of the IMI. Increase the initial challenge dose of *E. coli* resulted in faster immune response in primiparous cows (Vangroenweghe *et al*., 2004; Schukken *et al*., 2011), which the extend of induced cytokines synthesis, such as TNF-α and IL-8, in mammary epithelial cells positively correlated with the concentration of *E. coli* particles (Guntler *et al*., 2010). Congruently, expression of IL-8 and Interferon (IFN)-γ by milk somatic cells was increased in *E. coli* challenged mammary glands (Lee *et al*., 2003).

Buitenhuis *et al*. (2011) also described that in the early *E. coli* mastitis a large number of up-regulated transcripts were associated with immune response functions, mainly those involved in acute phase response, while the down-regulation transcripts were principally involved in fat metabolism, which is consistent with the milk fat content depression commonly observed during mastitis infection and later the up-regulated transcripts were associated with tissue healing processes and were independent of *E. coli* strain and dose and lactation stage and number. Another factor that should be considered is the linkage between lipid metabolism and inflammation, as the nuclear receptors known as Peroxisome Proliferator-Activated Receptors (PPARs) and Liver X Receptors (LXRs) that emerged as key regulators of lipid metabolism and inflammation (Lubick and Jutila, 2006; Bensinger and Tontonoz, 2008; Rios *et al*., 2008; Aitken *et al*., 2011; Moyes *et al*., 2010a; 2012b), as has been demonstrated in mammary glands infected with *Streptococcus uberis* (Moyes *et al*., 2010a).

The innate immune system represents the first line of defense in the host response to infection and is poised to immediately recognize and respond to the earliest stages of infection. The inherent capability of the innate system to respond to a vast number of pathogens is mediated by its ability to recognize highly conserved motifs shared by diverse pathogens, commonly referred to as PAMPs. It has been shown that a prompt response of the mammary after *E. coli* entry into the lumen of the gland is required to control the infection, which means that early detection of bacteria are of prime of importance (Bannerman *et al*., 2004; Porcherie *et al*., 2012).

The incidence and severity of septic *E. coli* mastitis in dairy cattle is mainly dependent on cow factors. During the periparturient period, the non-specific or innate immunity of the cow is depressed, which makes cows more susceptible to Intramammary Infection (IMI) by environmental pathogens like *E. coli*, while cows in mid lactation cure spontaneously from such infections (Burvenich *et al*., 2003; Mehrzad *et al*., 2005). There is substancial evidence indicating that at these periods, the expected influx of neutrophils, which form the first cellular defense against infection (Paape *et al*., 2003), into the mammary gland is delayed during inflammation after IMI with *E. coli* (Mehrzad *et al*., 2005; Schepper *et al*., 2007). In fact, the perturbations in neutrophil functions during early lactation are accompanied by modulation of TLR4 pathway genes, as diapedesis and migration process (Stevens *et al*., 2011).

Furthermore, Mehrzad *et al*. (2005) when classified cows as moderate and severe responders according to clinical symptoms and milk production output, observed an inverse relationship between pre-infection milk neutrophils microbicidal activity and CFU *E. coli* bacteria in milk, where the moderate cows the pre-infection milk and blood neutrophils microbicidal activity was about two fold higher than the severe cows.

Mammary epithelial cells challenged by *E. coli* bacteria must have the capacity to mountain a strong innate immune response in their own right and attract circulating immune effector cells such as neutrophils. The importance of these cells is demonstrated by their

role in the production of cytokines (Riollet *et al*., 2000; Strandberg *et al*., 2005; Griesbeck-Zilch *et al*., 2008; Guntler *et al*., 2011; Porcherie *et al*., 2012). The upregulation of cytokine production is a key component of the host innate immune response to infection (Bannerman *et al*., 2004; Schukken *et al*., 2011).

Regarding bovine Mammary Epithelial Cells (bMEC), Porcherie *et al*. (2012) showed that these cells are key players in initiating neutrophil inflammation during *E. coli* mastitis, as for instance, by the production of the chemotractic factor CXCL8 (IL-8). So, recognition of several PAMPs at a time could contribute to the onset of an early response of the cow after infection by *E. coli*. These authors showed that a repertorie of potential bacterial agonists can be sensed by bMEC and udder during *E. coli* mastitis, as which both bMEC and udder can express domain receptors for NOD1, NOD2, TLR1, TLR2, TLR4 and TLR6, but not hardly TLR5 and can act synergistically. So, LPS upon activation of TLR4 present a central role in the pathogenesis of clinical mastitis caused by this pathogen (Gonen *et al*., 2007) in a dose-dependent manner (Baumert *et al*., 2009). The inflammation caused by LPS also leads to alteration in milk parameters, as lactose and chloride levels, in dose dependent manners which are likely caused by greater tight junction damage by higher LPS doses (Werner-Misof *et al*., 2007). These parameters are also used to evaluated the indicators of inflammation in bovine mastitis and consequently in their diagnosis (Pyorala *et al*., 2003).

Lazard *et al*. (2011) demonstrated that neutrophil recruitment to the milk spaces is mediated through TNF-α, which is produced by alveolar macrophages in response to LPS/TLR4 signaling and is dependent on IL-8 and IL-1β signaling and regulated by iNOS-derived NO in a murine mastitis model. The ability to recruit cells into the mammary gland during the bacterial growth phase represent a crucial role since a 1 h delay in recruiting neutrophils can result in an 8-fold increase of *E. coli* (Hill, 1981). Both the MyD88 dependent and independent pathways in TLR4 signaling were activated in bMEC model (Ibeagha-Awemu *et al*., 2008). Despite the importance of LPS/TLR4 signaling pathway, Gonen *et al*. (2007) described that IMI of mice with *E. coli* P4 resulted in inflammation even in absence of LPS/TLR signaling. This inflammation response pointing out to additional factors beyond LPS and additional cells beyond alveolar macrophages play a role in the inflammatory response to *E. coli*. It has been suggested that in the absence of functional TLR4 the infecting *E. coli* P4 invaded epithelial cells with high efficiency, forming intracellular micro-colonies, since invasion of epithelial cells by *E. coli* is limited by alveolar macrophages using a process dependent on TLR4 signaling (Gonen *et al*., 2007; Elazar *et al*., 2010b; Schukken *et al*., 2011).

Infections caused by *E. coli* are more typically, but not exclusively, associated with fast and more dramatic immune response (Lee *et al*., 2003; Schukken *et al*., 2011). IMI with *E. coli* elicited systemic changes, including a febrile response and induction of acute-phase synthesis of LBP. In milk, this infection resulted in increased levels of insulin-like growth factor-1, IL-1β, IFN-γ, IL-12, IL-8, TNF-α, sCD14, LBP, the complement cleavage factor C5a, lactoferrin, lysozyme and lipid mediators, such as cyclooxygenase-2 and 5-lipoxygenase (Riollet *et al*., 2000; Bannerman *et al*., 2004; Schmitz *et al*., 2004).

Petzl *et al*. (2008) reported that *E. coli* inoculation in the mammary gland strongly upregulated the expression of β-defensins, TLR2 and TLR4 in the pathogen inoculated udder quarters, as well as, in mammary lymph nodes. In constrast, *S. aureus* did not significantly regulate the expression of these genes during the first 24 h after pathogen inoculation. Only 84 h after inoculation, the expression of β-defensins, but not of TLRs was significantly upregulated (<20 fold) in *S. aureus* inoculates mammary glands.

E. coli IMIs induce distinct local and systemic transcriptome responses in the mammary gland. The local response, only in infected quarters, mainly involved in immune response and inflammation, while the systemic reactions, in both infected and neighboring quarters, comprises antigen processing and presentation, cytokines, protein degradation and apoptosis. Enhanced expression of antimicrobial genes, acute phase genes and indicators of oxidative stress point out to an active defense reaction in infected and neighboring healthy quarters (Mitterhuemer *et al*., 2010).

In this concern, data support an important sentinel function for teats, as these tissues respond rapidly and intensively, with production of cytokines and antimicrobial peptides. For example, genomic analysis at 12 h post-infection with *E. coli* the inflammatory response was greatest in teat cistern and gland cistern. Only 24 h post-infection, the lobulo-alveolar region responds, at the time the inflammatory response was greatest of all regions (Rinaldi *et al*., 2010).

1.3. Mastitis Caused by *Staphylococcus Aureus*

S. aureus mastitis remains a worldwide problem for the dairy industry and producers and can cause both subclinical and clinical mastitis (Barkema *et al*., 2006), which severity and outcome of infection depend, in part,

on strain-factors (Marechal *et al*., 2011) and cow factors (Barkema *et al*., 2006). The cure rate of antimicrobial treatments for this agent is low and, therefore, the disease has not been effectively eliminated and/or controlled in many herds (Barkema *et al*., 2006). Staphylococcal infections are characterized by an ability to colonize the mammary tissue and survival of the bacterial inside epithelial cells, macrophages and even neutrophils (Gresham *et al*., 2000; Hebert *et al*., 2000; Lowy, 2006).

It is commonly assumed that most IMI are result of cow-to-cow transmission, however other sources of *S. aureus* bacteria in the environment of dairy cow have been described. Presumably, contagious strain of *S. aureus* co-exists with a large collection of non-contagious strains (Zadoks *et al*., 2002). Haveri *et al*. (2007; 2008) compared bacterial genomics of strains from persistent infections and form transient infections and found that genetic elements such as clonal type and penicillin resistance were over-represented in *S. aureus* isolated from persistent IMI. This microorganism is characterized by dynamic fluctuations and cyclic bacterial shedding in milk, which leads to fluctuations in milk SCC that normally fluctuate depending on organism's number and viability (Schukken *et al*., 2011; Souza *et al*., 2012).

In contrast to *E. coli* mastitis, *S. aureus* mastitis is characterized by a more moderate and delayed SCC increase, due in part, to limited cytokine response (Bannerman *et al*., 2004). Congruently, Riollet *et al*. (2000) also described no detection of IL-1β, TNF-α, IL-8, bovine serum albumin, in milk whey from *S. aureus* infected animals. Indeed, the ability of milk to generate the complement cleavage product C5a in whey samples after addition of zymosan through complement activation was evaluated and *E. coli* lead to a huge augment of C5a production (up to 100-fold), in contrast to a much less production in whey milk from *S. aureus* infected animals. Rainard *et al*. (2008) also showed that LTA from *S. aureus* induced an increase in chemokine and IL-1β, but little TNF-α in the milk.

Although, *S. aureus* is regarded as a gram-positive bacteria, the expression of TLR2 were correlated with TLR4, indicating coordinating regulation of these two PRRs (Goldammer *et al*., 2004; Ibeagha-Awemu *et al*., 2008), although the expression of TLR9 was not increased in mastitis (Goldammer *et al*., 2004).

Cytokine gene expression in mammary epithelial cells induced by *S. aureus* infection was delayed and less than 5% of the cytokine expression observed in experiment of *E. coli* (Lee *et al*., 2003; Yang *et al*., 2008; Guntler *et al*., 2010). This impaired proinflammatory activation is paralleled by a complete lack of NF-κB activation in primary bovine mammary epithelial cells by *S. aureus* or LTA. In contrast to *E. coli* and LPS that activates strongly NF-κB in these cells. A large proportion of this activation is attributable to TLR-mediated signaling, since dual transdominant negative DN-MyD88-DN-TRIF factor blocks more than 80% of the pathogen-related NF-κB activation in primary bovine mammary epithelial cells. These facts may contribute to well-known ability of this bacterium to establish chronic intramammary infections (Yang *et al*., 2008; Guntler *et al*., 2011).

For example, the Interleukin (IL)-8 and TNF-α were not detected in milk from quarters experimentally infected with *S. aureus* (Riollet *et al*., 2000; Bannerman *et al*., 2004), although, the mRNA expression of TNF-α in mammary cells increases during infection (Alluwaimi *et al*., 2003). Expression of IL-8 by milk somatic cells was also increased in *S. aureus* challenged mammary glands, but in lower magnitude than *E. coli* challenged mammary glands. Although, the expression of IFN-γ was not increased in milk somatic cells from *S. aureus* challenged quarters (Lee *et al*., 2003).

In vitro, mammary epithelial cells demonstrated greater mRNA expression of IL-1β, IL-8 and TNF-α 24 h after infection with *E. coli* than *S. aureus* (Lahouassa *et al*., 2007). Wellnitz *et al*. (2011) also found that infusion of *E. coli* LPS induced an increased TNF-α in milk from glands given LPS, but not by *S. aureus* LTA. The levels of lactate dehydrogenase, an enzyme released by degenerating cells, was greater in milk from glands instilled with LPS than with LTA. LPS was also a stronger induced of IL-8 and IL-1β.

Conversely, the ability of to induce clinical or subclinical mastitis was dependent on the dose used. LTA proved to induce strongly the secretion of the chemokines CXCL1, CXCL2, CXCL3 and CXCL8, which induced neutrophils recruitment. The complement-derived chemoattractant C5a was generated in milk only with the highest dose of LTA used. Furthermore, the pro-inflammatory cytokine IL-1β has been induced in milk, but there is few amount of TNF-α and no IFN-γ (Rainard *et al*., 2008).

The Muramyl Peptide (MDP), an elementary constituent of the bacterial peptidoglycan, induce a prompt influx of neutrophils mediated by chemoattractants for these leukocytes (CXCL1, CXCL2, CXCL3, CXCL8 and C5a) and the highest concentrations of these chemoattractants were followed after challenge in combination with LTA, which signal transduction is mediated by TLR2, although they not

contribute significantly to pro-inflammatory cytokines. Thus, TLR2 and NOD2, a major sensor for MDP, pathways could cooperate to trigger an innate immune response to *S. aureus* mastitis (Bougarn *et al*., 2010).

Induction of immune functions in mammary epithelial cells is accomplished via the activation of the relevant TLR and their downstream signaling pathways. Induction of these genes by *S. aureus* is reduced, due to, in part, impairment of MyD88 signaling, immediately downstream from trans-membrane TLR (e.g., TLR2, TLR4). *S. aureus* apparently prevents the formation of so-called Myddossome around TIR domain of the TLR forming the structural plataform for the attachment of further downstream acting factors (Motshwene *et al*., 2009; Lin *et al*., 2010; Schukken *et al*., 2011). As a consequence, *S. aureus* elicits an immune response in these cells dominantly by IL-6, while *E. coli* also activates IL-1β and TNF-α (Guntler *et al*., 2011; Schukken *et al*., 2011). The upregulation of IL-6 by both bacteria may be due to a MyD88 independent mechanism (Guntler *et al*., 2010; 2011), which as cited above is associated with late-phase NF-κB response.

It has also been suggested that *S. aureus* impaired NF-κB activation in mammary epithelial cells resulting in very low cytokine expression (Lara-Zarate *et al*., 2011). These authors reported that bovine prolactin stimulates *S. aureus* internalization in bovine mammary gland by regulating several innate immune elements, which is often modulated by NF-κB. On the other hand, prolactin induced NF-κB activation in bovine mammary epithelial cells; however, it was inhibited by *S. aureus* in presence of this hormone. When, these authors blocked NF-κB activation with acetylsalicylic acid, an inhibition of *S. aureus* internalization was found (48%) in prolactin stimulated cells. The infection of bovine mammary epithelial cells with *S. aureus* induced inhibition of NF-κB activation in the presence of prolactin that correlates with down regulation in prolactin-mediated TNF-α (27%) and nitric oxide production in mammary epithelial cells.

Curiously, Griesbeck-Zilch *et al*. (2008) encountered differences in expression of TLR2 and TLR4 by mammary epithelial cells in *S. aureus* and *E. coli* infections only after 24 h, when *S. aureus*-induced expression was significant lower. In contrast, after 1 h *S. aureus* induced a significantly higher expression level of TNF-α and IL-1β, but after 6 and 24 h the transcription activity in *E. coli* treated cells was higher. In contrast, *E. coli* induced a significant increase expression of IL-8 after 1h, but *S. aureus* caused no alteration in this chemokine. The Regulated upon Activation, Normal T-Cell Expressed and Secreted (RANTES) increased in *S. aureus* and *E. coli* treated bovine mammary epithelial cells after 1 h, whereas after 6 and 24 h the expression was significantly higher in *E. coli* treated cells. Lactoferrin showed a deviating expression pattern to pathogen stimulation, in which at 1 h *E. coli* induced a higher mRNA expression, whereas the highest level was reached after 24 h of *S. aureus* stimulation. The complement factor 3 was the only factor that responded equally to both microorganisms.

Genini *et al*. (2011) described that mastitis induced a prominence of metabolic and stress signals in the early stage and of the immune response and lipid metabolism in the late stage, both mechanisms apparently modulated by few genes. Comparison of *E. coli* and *S. aureus* infections in cattle revealed that affected genes showing opposite regulation had the same altered biological functions and provided evidence that *E. coli* caused a stronger host response. The majority of genes with opposed regulation associated with immune response belong to antigen presentation, inflammatory response, cell-to-cell signaling and interaction network. Both cell death and lipid metabolism were among the most significant molecular functions altered in proteins of cows infected with either *E. coli* or *S. aureus*.

After 48 h post-challenged with *S. aureus* TLR1 was significantly expressed in ductal, gland cistern and teat canal, TLR3 showed a moderate increase in teat canal tissue, TLR6 and TLR7 presented a moderate increased in gland cistern tissue, TLR5 and TLR7 were also significantly increased in alveolar in alveolar tissue. Conversely, the genes encoding TLR4, NOD1 and NOD2 were significantly decreased in teat canal tissue, TLR6 in ductal tissue and TLR8 in gland cistern tissue. TLR2, TLR9 and TLR10 showed no differential expression across these four tissues regions. Of the regions examined, chemokine and effector molecule expression was most significantly stimulated in alveolar tissue, in particular the expression of serum amyloid A and haptoglobulin, two acute phase proteins and defensins-β 4 and 5 (Whelehan *et al*., 2011).

Thus, *S. aureus* appears to mostly circumvent the host immune response and IMI typically result in a very moderate host response with minimal observable innate immune response (Bannerman *et al*., 2004; Bannerman, 2009; Petzl *et al*., 2008; Schukken *et al*., 2011).

1.4. Therapeutic Opportunities

CD14 either in membrane or in soluble form (sCD14) is a high-affinity protein for the complex of bacterial LPS and LPS-LBP protein and thus interact with TLR4 in LPS signaling (Medzhitov, 2001; Takeda *et al*., 2003; Nemchinov *et al*., 2006). Cells lacking mCD14, such as

endothelial and epithelial cells, utilize sCD14 present in serum and milk to aid in LPS recognition by TLR4 (Aitken *et al*., 2011). Binding of soluble form of CD14 to LPS, found in the outer of *E. coli*, enhances the innate immune responses, reduces the severity of mastitis and facilitates clearance and neutralization of LPS, thus preventing the development of endotoxic mastitis. Thus, Lee *et al*. (2003) found that the infusion of recombinant bovine sCD14 lead to an increase in SCC, due to more rapid recruitment of neutrophils that was accompanied by a faster clearance of bacteria, lower concentration of TNF-α and IL-8 in milk and milder clinical symptoms. Congruently, Nemchinov *et al*. (2006) demonstrated that the recombinant bovine CD14 receptor produced in plants reduced the severity of *E. coli* mastitis, leading to enhancement of LPS-induced neutrophil recruitment, lower numbers of viable bacteria in milk resulting in absence of clinical symptoms.

Kauf *et al*. (2007) in attempt to heighten the inflammatory response during *S. aureus* intramammary infection infused LPS in quarters experimentally infected with *S. aureus*. They found an increase in SCC in quarters between 24 and 72 h post LPS-infusion, as well as, an increase in bovine serum albumin in milk, which reflect alterations in vascular permeability and are indicator of udder quarter inflammation, between 4-48 and 480 h post LPS-infusion. There was no detection of TNF-α in *S. aureus*-infected quarters administrated PBS at any time during the study. Conversely, infected quarters infused with LPS showed an increased TNF-α concentrations in milk between 4-8 h post LPS-infusion. Moreover, a trend toward a lower recovery of viable bacteria from LPS- versus PBS-infused quarters between 4-13 h post LPS-infusion was observed. Interestingly, this trend occurred in the inflammatory responses elicited by LPS. Subsequent *in vitro* inoculation of milk obtained from udder quarters infused with LPS or PBS demonstrated that the growth of *S. aureus* in milk from LPS-infused udder was significantly inhibited and an overall negative correlation existed between milk SCC and *in vitro S. aureus* growth in milk inoculated with *S. aureus* and incubated for 6 or 12 h.

In a mastitis rat model, the infusion of CpG-DNA in mammary glands stimulated the secretion of IL-6 and TNF-α at different points, reduced viable *S. aureus* (Zhu *et al*., 2007a) and *E. coli* (Zhu *et al*., 2008) in mammary tissues, decreased the activity of NAGase, promoted the expression of TLR9 and induced more rapid infiltration of neutrophils to mammary tissue at initial stages of experimentally induced mastitis induced in rat model (Zhu *et al*., 2007a; 2008). In goats, Zhu *et al*. (2007b) demonstrated that the infusion of CpG-DNA in the mammary glands induced a decrease in viable *E. coli*, reduced bacteria counts in milk, promoted the expression of TLR9, stimulated the production of IL-6, attenuated the impact of inflammation mediators on cells and significantly shortened the inflammation course.

The retinoid, a group of derivates of vitamin A, exert various immunomodulatory actions. It has been demonstrated that the administration of retinoid acids protects rats against neutrophil-induced oxidative stress in acute experimental mastitis (Gu *et al*., 2009a; 2009b). A mechanism by which this protection is conferred is through TLR4. Gu *et al*. (2010) found that TLR4 gene expression reached its peak earlier in retinoid acid-treated rats and that retinoid acid decreased NF-κB DNA binding activity and the level of IL-1β protein expression in mammary gland. So, retinoid acid leads to attenuation of LPS-induced inflammation response by repression of TLR4/NF-κB signaling system. Another mechanism that can also be involved was demonstrated by Uematsu *et al*. (2008) who found that retinoid acids, in a dose-dependent manner, regulated the differentiation of interleukin 17-producing T helper cells, which in turn mediated neutrophil response (Schukken *et al*., 2011).

Pheromonicin-SA (Ph-SA) is an engineered multidomain bactericidal peptide (Qiu *et al*., 2003) that has effect against *S. aureus*. Zhu *et al*. (2012) when exposed primary mammary epithelial cells to Ph-SA found that this compound increases the expression of TLR2, TNF-α, IL-1β, IL-8 and lactoferrin and later the expression of TLR4. Thus, Ph-SA may be value as an antimicrobial in promoting innate immune response by *S. aureus aureus*-infected bovine mammary epithelial cells, especially regarding the inhibition of innate immune response induced by *S. aureus* which leads to the chronification of the inflammatory response (Bannerman *et al*., 2004; Lahouassa *et al*., 2007; Yang *et al*., 2008; Motshwene *et al*., 2009; Guntler *et al*., 2010; Lin *et al*., 2010; Lara-Zarate *et al*., 2011; Schukken *et al*., 2011; Wellnitz *et al*., 2012).

TLR signaling induces 25-hydroxyvitamin D3 1α-hydrolase expression in macrophages. The 25-hydroxyvitamin D_3 1α-hydrolase is the primary enzyme that converts 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3, the active vitamin D_3 metabolite. It was shown that the expression 25-hydroxyvitamin D3 1α-hydrolase was significantly increased in tissue and cells from of infected mammary glands and was predominantly expressed in $CD14^+$ cells (Nelson *et al*., 2010), which is expressed in both neutrophils and macrophages in milk (Paape *et al*., 1996). Thus, regarding the importance of innate immunity for mammary gland health (Paape *et al*., 2003; Rainard and Riollet, 2006; Elazar *et al*., 2010a; 2010b), efforts to find

optimal range of 1, 25-dihydroxyvitamin D_3 concentrations for proper immune function in cattle has implications for bovine health.

2. CONCLUSION

The innate immunity is crucial to maintain mammary gland healthy which is mediated through recognition of Pattern Recognition Receptors (PRRs). The PRRs recognized specific patterns of microbial components that are conserved among pathogens known as Pathogen-Associated Molecular Patterns (PAMPs). The interaction of PRRs and PAMPs mediated the inflammatory response characterized by each mastitis-causing pathogen that can contribute to the development of severe acute inflammation or chronic mastitis.

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