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Research paper

Host-response patterns of intramammary infections in dairy cows

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ABSTRACT

Many different bacterial species have the ability to cause an infection of the bovine mammary gland and the host response to these infections is what we recognize as mastitis. In this review we evaluate the pathogen specific response to the three main bacterial species causing bovine mastitis: *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus*. In this paper we will review the bacterial growth patterns, host immune response and clinical response that results from the intramammary infections. Clear differences in bacterial growth pattern are shown between bacterial species. The dominant pattern in *E. coli* infections is a short duration high bacteria count infection, in *S. aureus* this is more commonly a persistent infection with relative low bacteria counts and in *S. uberis* a long duration high bacteria count infection is often observed. The host immune response differs significantly depending on the invading bacterial species. The underlying reasons for the differences and the resulting host response are described. Finally we discuss the clinical response pattern for each of the three bacterial species. The largest contrast is between *E. coli and S. aureus* where a larger proportion of *E. coli* infections cause potentially severe clinical symptoms, whereas the majority of *S. aureus* infections go clinically unnoticed.

The relevance of fully understanding the bovine host response to intramammary infection is discussed, some major gaps in our knowledge are highlighted and directions for future research are indicated.

1. Introduction

Intramammary infections (IMI) in dairy cows are a major concern for the dairy industry. These infections lead to severe milk loss are potentially fatal, and are a major cost to dairy farmers. For this reason, there is an active research effort to understand the pathogenesis of mastitis, the inflammatory response to an intramammary infection. In the last decade our knowledge about the inflammatory response to infection has improved, both in terms of a better understanding of the mammalian immune response and the immune response of the bovine mammary gland

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(e.g. Burton and Erskine, 2003; Rainard and Riollet, 2006). Similarly, biopsies of the mammary epithelium have revealed much about the regulation of genes involved in the host response to an IMI (Buitenhuis et al., 2011; Moyes et al., 2009; Mitterhuemer et al., 2010; Genini et al., 2011). The immune response pattern in the acute phase response was dominated by an up-regulation of chemokine and cytokine pathways, Toll-like receptor signalling pathways and leukocyte transendothelial migration (Buitenhuis et al., 2011). The importance of the innate arm of the immune defence has been more fully appreciated with our increased understanding of the interaction of specific conserved pathogen associated molecular patterns (PAMPs) with specific receptors on host cells that recognize these PAMPs. Toll-like receptors (TLRs) are host cell sensors that are recognizing conserved pathogen-associated molecular patterns. The Toll-like receptors such as Toll-like receptor 4 (TLR4) and TLR2 are important pattern recognition receptors that recognize conserved microbial molecules such as LPS as PAMPs associated with gram-negative bacterial infections, whereas lipoteichoic acid (LTA), is now recognized as a pathogen-associated molecular pattern of at least some of the gram-positive bacteria (Rainard et al., 2008).

Important differences exist in the response to IMI caused by different bacterial species. Bacterial growth patterns and the associated innate immune response differ significantly between gram-negative bacteria such as *Escherichia coli* (*E. coli*) and gram-positive bacteria such as *Streptococcus uberis* (*S. uberis*). Infections caused by *E. coli* are more typically, but not exclusively, associated with a fast and more dramatic immune response, whereas infections with *S. uberis* are characterized by a delayed and less dramatic response (Bannerman, 2009; Rambeaud et al., 2003; Genini et al., 2011). In contrast, *Staphylococcus aureus* (*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 (Petzl et al., 2008; Bannerman, 2009). These pathogen specific responses can also be recognized in the somatic cell count patterns in milk relative to IMI, milk production losses and risks of culling and death (deHaas et al., 2004; Bar et al., 2008; Gröhn et al., 2004).

A full understanding of the adaptive immunity in the context of mammary health provides challenges since the ruminant mammary gland is unique in that lymphocyte trafficking, which is essential to adaptive immunity, is shared with the peripheral immune system rather than the common mucosal immune system. Protective immunity of the bovine mammary gland invoked by natural infection with bacterial organisms has shown to be relatively short-lived. A partial protection against subsequent natural infection disappeared within weeks (Suojala et al., 2008; Schukken et al., 2009). This relative inability to mount an adequate and long-lasting protective response to natural infection provides a major challenge for the development of effective vaccines protecting the bovine mammary gland from infection.

In this review we aim to describe the bacterial growth patterns of the major bacterial pathogens causing mastitis (*S. uberis*, *S. aureus* and *E. coli*), discuss our current understanding of the innate and adaptive host response patterns to these bacterial intramammary infections and the observable clinical response of the dairy cow to IMI with these organisms. We focus on information that has become available in the last decade. Our ultimate goal is to provide insight into the interplay of pathogen and host as they interact in the mammary gland such that potential preventative or curative actions can be taken to eliminate infection and minimize the damage due to inflammation.

2. Bacterial growth patterns

2.1. E. coli

Gram-negative bacteria play a considerable role in bovine mastitis. The most important and best investigated species is *E. coli*. Together with *Klebsiella* spp. and *Enterobacter* spp., it belongs to the group of coliforms. Further frequently isolated gram-negative mastitis pathogens are *Serratia* spp., *Pseudomonas* and *Proteus* (Hogan and Smith, 2003). Occasional cases of bovine mastitis caused by *Pasteurella*, *Acinetobacter* and *Salmonella* have been reported (Smith et al., 1989; Malinowski et al., 2006). Gram-negative bacteria are generally considered environmental pathogens however contagious behavior (transfer from cow to cow) of these pathogens has been proposed (Munoz et al., 2007). Several authors proposed that specific *E. coli* strains are more adopted to the mammary gland (Shpigel et al., 2008; Dogan et al., 2006; Döpfer et al., 1999), whereas others maintain that all *E. coli* bacteria are equally likely to cause bovine mastitis (Suojala et al., 2011; Burvenich et al., 2003). Natural reservoirs of coliform bacteria are the gastrointestinal tract, soil and bedding material.

Pathogenicity characteristics of gram-negative mastitis pathogens have been studied in recent years. It was shown that *E. coli* pathogens express a variety of virulence factors but no coherence between the severity of the disease and specific virulence factors could be defined (Bean et al., 2004; Wenz et al., 2006; Suojala et al., 2011). However, the ability to grow in mammary secretions and to liberate lipopolysaccharide (LPS) is crucial in the pathogenesis of mastitis caused by gram-negative bacteria. The faster bacterial numbers increase in the mammary gland, the more LPS is present in the mammary gland and the faster the inflammatory response and clinical disease may occur (Mehrzad et al., 2008). Gram-negative bacteria utilize milk nutrients to grow and multiply. A clear advantage for the gram-negative bacteria is the utilization of lactose as an energy source from milk. Under laboratory conditions, *E. coli* can multiply with a minimum generation time of 10.6 min in standard broth and a generation time of 15.2 min in milk growing up to a concentration of eventually 10^9 cfu/ml (Goldberg et al., 1994). In experimental challenge trials the multiplication of *E. coli* and other gram-negative mastitis pathogens has been studied. *E. coli*, *Klebsiella* and *Enterobacter* are capable of multiplying under near anaerobic conditions growing up to 10^8 cfu/ml of milk (Hogan et al., 1992). Non-lactose fermenting gram-negative bacteria such as *Serratia* and *Pseudomonas* show comparatively lower bacterial counts in milk. Bannerman et al. (2005) showed maximum values of 10^4 cfu/ml after experimental infection with *Pseudomonas aeruginosa* after

16 h (Bannerman et al., 2005) and *Serratia marcescens* after 12 h. Overall data of maximum bacterial counts in milk differ between studies probably due to different conditions in experimental trials. Important factors affecting intramammary bacterial growth are the stage of lactation of the cow, the initial amount of leukocytes in milk and the presence of soluble antimicrobial factors. Sensing the pathogen and initiating an immune response depends on the initial number of bacteria present at the start of the IMI. Increasing the initial challenge dose of *E. coli* resulted in a faster immune response in primiparous cows (Vangroenweghe et al., 2004). A causal mechanism for the relationship between initial bacterial numbers and subsequent immune response was recently identified by demonstrating that the extent of induced cytokine synthesis (TNF-α, IL-8) in mammary epithelial cells (MEC) positively correlated with the concentration of *E. coli* particles (Günther et al., 2010). Hence, MECs appear to titrate the quantity of invading *E. coli* and calibrate the cytokine expression accordingly.

2.2. S. aureus

S. aureus is an important cause of IMI in dairy cows. It is commonly assumed that most IMI are the result of cow-to-cow transmission where other infected animals in the herd are the source of the organism. However, other sources of *S. aureus* bacteria in the environment of a dairy cow have been described and in many herds a dominant, presumably contagious strain of *S. aureus* co-exists with a large collection of other, presumably non-contagious strains (Zadoks et al., 2002). Both in experimentally infected cows and in cows sampled longitudinally with a naturally occurring *S. aureus* IMI a low and high shedding cycle were observed. A low shedding cycle was defined as shedding a mean of <1000 cfu/ml and high shedding cycle was defined as a mean cfu of >2000 cfu/ml. Both low and high shedding IMI were characterized by a cyclical shedding pattern (Sears et al., 1990). In all IMI shedding of *S. aureus* was persistent and the infection remained present in the infected quarter for the duration of the study (>20 days). Several other investigators have observed similar infection patterns with *S. aureus* typically associated with long term intramammary persistence and bacteria counts that were much lower than the counts observed in *E. coli* and *S. uberis* IMI. Haveri et al. (2007, 2008) compared bacterial genomics of strains from persistent infections and from transient infections. Their results indicated that genetic elements such as clonal type and penicillin resistance were over-represented in *S. aureus* isolated from persistent IMI.

2.3. S. uberis

S. uberis is a common cause of clinical and subclinical infection. Infections with *S. uberis* are generally considered of environmental nature however contagious behavior (transfer from cow to cow) of these pathogens is frequently observed (Zadoks, 2007). Natural reservoirs of *S. uberis* are the gastrointestinal tract, skin, soil and bedding material (Zadoks et al., 2005). Following experimentally induced infection of the lactating mammary gland, *S. uberis* is found predominantly in the lumenal areas of secretory alveoli and ductular tissue. Bacterial numbers can reach up to 10^9 cfu/ml of milk but, typically, clinical disease is apparent at bacterial concentrations of 10^6 to 10^8 cfu/ml (Leigh et al., 2010). Given the ability of *S. uberis* to colonize other body sites prior to infection of the mammary gland, it can be postulated that *S. uberis* isolated from clinical disease represent a specialized subset of the total population (Zadoks, 2007; Ward et al., 2009; Leigh et al., 2010). In support of this, recent analysis revealed clusters of sequence types (STs) that were associated with disease and other STs that were only isolated from mammary glands that did not show signs of disease (Tomita et al., 2008). Furthermore, strains of *S. uberis* that differ in virulence for the lactating udder have been described following challenge under experimentally controlled conditions (Hill, 1988; Smith et al., 2003; Leigh et al., 2010). Strain 0140J caused infection and disease in 16 of 18 challenged quarters, whereas strain EF20 colonized all animals only transiently at low level and caused mild signs of disease in only 2 of 18 challenged quarters (Hill, 1988). More recently, this difference in ability to colonize the mammary gland and cause clinical disease was associated with the presence of sortase anchored proteins (Leigh et al., 2010). As neutrophils constitute one of the major defences of lactating gland, it is likely that inhibition of their function is of vital importance to the outcome of *S. uberis* infection. The bacterial molecules secreted by *S. uberis* that are capable of inhibiting neutrophil bactericidal function have not been identified (Field et al., 2003). Inhibited neutrophils failed to produce pseudopodia, became rounded and showed an altered distribution of actin (Field et al., 1997).

2.4. Persistence of infection

Persistent intramammary infections are an important component of the problem in bovine mastitis. Clinical mastitis with possible life-threatening severity is of importance to the cow and the dairy farmer, however the presence of persistent intramammary infections causing long-term increases in somatic cell counts and repeated clinical cases forms another major concern to dairy producers. Persistent infections are very common for gram-positive organisms such as *Streptococcus agalactiae*, *S. aureus*, CNS and *Corynebacterium bovis*; are common for gram-positive pathogens such as *S. uberis* and *Streptococcus dysgalactiae* and are not uncommon in gram-negative bacteria such as *Serratia* spp., *Klebsiella* spp. and have also been reported for *E. coli* IMI. *Mycoplasma* spp. also consistently causes persistent IMI. In several studies on persistent intramammary infections, it was observed that bacterial strains causing persistent infections are different compared to strains causing transient infections. These strain differences were not only observed using descriptive molecular typing techniques (Zadoks et al., 2003; Haveri et al., 2007; Munoz et al., 2007; Leigh et al., 2010), but also important phenotypic differences were observed in in vitro functional assays. These functional assays included bacterial adhesion and invasion into epithelial cells (Dogan et al., 2006; White et al., 2010) and bacterial growth dynamics in mammary macrophages (Dogan et al., 2006). These observations gave rise to the hypothesis that bacterial adaptation to the

bovine mammary gland is an active and ongoing selection process across all major mastitis causing bacterial species (Shpigel et al., 2008). Host-adaptation of bacterial species would result in more persistent infections that are associated with less clinically severe disease patterns and more opportunity for persistence and transmission of dominant strains of a bacterial species in the herd.

3. Immune response patterns

The immune response is often described as consisting of an innate and adaptive immune response arm. The adaptive immune system is the arm of the immune system that specifically responds to an antigen. Whereas the innate immune system uses either passive barriers or receptors that recognize conserved microbial molecules.

3.1. Innate response

3.1.1. Toll-like receptors and ligands

The innate defence mechanisms of the mammary gland include physical barriers such as the teat sphincter, chemical barriers such as teat canal keratin and lactoferrin, and more proper components of the immune system such as macrophages, dendritic cells, mast cells, neutrophils, eosinophils and natural killer (NK) cells (Werling et al., 2006; Riollet et al., 2000). Perceiving the presence of a pathogen is the first and mandatory step in the immune defence against the invading pathogen. Mammals are equipped with a battery of trans-membrane receptors sensing the presence molecular components of pathogens. The 13 different mammalian TLRs represent the best-described family of such 'pattern recognition receptors' (PPRs) (Akira et al., 2006; Akira and Takeda, 2004; Ozinsky et al., 2000). Some of these receptors span across the cell membrane and bind bacterial ligands with their extracellular domain. TLR2, for example, is known to bind LTA from some but not all of the gram-positive bacteria (Schwandner et al., 1999; Pedersen et al., 2010). For example, *S. uberis* and *S. agalactiae* were surprisingly found as failing to activate TLR2 (Farhat et al., 2008). In the lipopolysaccharide molecule, protein A is the relevant ligand for TLR4 (Hirschfeld et al., 2001). The currently known Toll-like receptors, their ligand and ligand location are summarized in Table 1. The discovery of Toll-like receptors as evolutionary conservative molecules and their role in innate immune response has opened a new area of study to better understand the primary immune response in the mammary gland. The identification of CD14, TLR2 and TLR4 on milk fat globule membranes suggests a direct role for the mammary gland parenchyma in pathogen detection. Biopsies of the parenchyma of udders with *E. coli* mastitis show increased mRNA abundance of the TLR2 and TLR4 genes (but not of TLR9) (Goldammer et al., 2004). These pathogen-receptors are expressed in MECs and infection strongly induces their expression (Petzl et al., 2008).

All TLR signal transduction pathways are known to eventually 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 (Fig. 1). These NF-κB factors may 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 this factor complex acts as a main switch to orchestrate a whole battery of immune defence genes. In fact, several of the pro-inflammatory cytokines and adhesion molecules that mediate the localized and/or systemic responses to gram-negative mastitis, including IL-1β, IL-6, IL-8, and TNF-α, are up-regulated by LPS in an NF-κB-dependent manner (see cytokine profiles below). Cytokine expression in cows with mastitis has been shown to correlate with NF-κB activation. Biological effects induced by LPS are primarily mediated by the cytokines TNF-α, IL-1 and IL-6, which are produced by mononuclear phagocytes and known to be involved in septic shock, and by IL-8, which is a chemoattractant for PMN and granulocyte-activating cytokines. This was confirmed by Elazar et al. (2010a) who demonstrated the importance of TLR4 in PMN migration after intramammary LPS challenge. Elazar et al. (2010a) described that upon intramammary challenge with LPS, an increased PMN migration was demonstrated in TLR4 wild type mice, whereas in TLR4-deficient mice, LPS failed to induce PMN migration. A study by Petzl et al. (2008) showed that indeed intramammary *E. coli* inoculation strongly and significantly up-regulated the expression of β-defensins, TLR2 and TLR4 in the pathogen inoculated udder quarters as well as in mammary lymph nodes. In this study TLR3 and TLR6 were not significantly regulated by the infections. Immunohistochemistry identified mammary epithelial cells as sites for the up-regulated TLR2 and β-defensin expression. *S. aureus*, in contrast, did not significantly regulate the expression of any of these genes during the first 24 h after pathogen inoculation (Petzl et al., 2008; Yang et al., 2008). Griesbeck-Zilch et al. (2008) confirmed this finding, and showed a significant lower TLR4 mRNA level in *S. aureus* infected primary MECs compared to *E. coli* infected cells. In an in vitro infection experiment, Yang et al. (2008) showed that absence of TLR-activation through ligand binding from the pathogenic *S. aureus* strain was not the cause for the inadequate mammary immune response elicited by this pathogen. Inactivated particles from both *E. coli* and *S. aureus* activated TLR2 and TLR4 in a similar manner in the HEK293 reconstitution system of TLR signal transduction. Rather, *S. aureus* impaired NF-κB activation in MECs resulted in a very low cytokine expression as measured by mRNA quantity (Lara-Zárate et al., 2011). Cytokine gene expression in *S. aureus* infection was delayed and less than 5% of the cytokine expression observed in a comparative experiment with *E. coli* (Yang et al., 2008; Günther et al., 2010). Cytokine expression in mammary epithelial cells exposed to heat treated *E. coli* bacteria was clustered in a regulatory network with TNF-α and IL-1 in a central position, in contrast these cytokines were barely up-regulated in an identical setting with *S. aureus*. Both bacteria showed an up-regulation in IL-6 which may be due to a MyD88 independent mechanism (Günther et al., 2010). Particularly in cells co-cultured with *S. aureus* an up-regulation of IFN-β was observed, again likely due to a MyD88 independent NF-κB activation (Günther et al., 2011).

Table 1
Toll-like-receptors and their ligands.

Receptor	Ligand	Ligand location	Reference
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria	Takeuchi et al. (2002)
	Soluble factors	*Neisseria meningitidis*	Wyllie et al. (2000)
TLR2	Lipoproteins/lipopeptides	Different bacterial pathogens	Aliprantis et al. (1999)
	Peptidoglycan	Gram-positive bacteria	Takeuchi et al. (1999), Schwandner et al. (1999)
	Lipoteichoic acid	Gram-positive bacteria	Schwandner et al. (1999)
	Lipoarabinomannan	Mycobacteria	Means et al. (1999)
	Phenol-soluble modulin	*Staphylococcus epidermidis*	Hajjar et al. (2001)
	Glycoinositol phospholipids	*Trypanosoma cruzi*	Coelho et al. (2002)
	Glycolipids	*Treponema maltophilum*	Opitz et al. (2001)
	Porines	*Neisseria*	Massari et al. (2002)
	Atypic lipopolysaccharides	*Leptospira interrogans*	Werts et al. (2001)
	Atypic lipopolysaccharides	*Porphyormonas gingivalis*	Hirschfeld et al. (2001)
	Zymosan	Fungi	Underhill et al. (1999)
	Heat-shock protein 70	Host cells	Asea et al. (2002)
TLR3	Double-stranded RNA, poly I:C	Viruses	Alexopoulou et al. (2001)
TLR4	Lipopolysaccharide	Gram-negative bacteria	Poltorak et al. (1998)
	Taxol	Plants	Kawasaki et al. (2000)
	Fusion protein	Respiratory syncytial virus	Kurt-Jones et al. (2000)
	Envelope protein	Mouse mammary tumour virus	Rassa et al. (2002)
	Heat-shock protein 60	*Clamydia pneumoniae*	Bulut et al. (2002), Ohashi et al. (2000)
	Heat-shock protein 70	Host cells	Vabulas et al. (2002)
	Fibronectin	Host cells	Okamura et al. (2001)
	Hyaluronic acid	Host cells	Termeer et al. (2002)
	Heparan sulfate	Host cells	Johnson et al. (2002)
	Fibrinogen	Host cells	Smiley et al. (2001)
TLR5	Flagellin	Bacteria	Hayashi et al. (2001)
TLR6	Diacyl lipopeptides	*Mycoplasma*	Takeuchi et al. (2001)
	Lipoteichoic acid	Gram-positive bacteria	Schwandner et al. (1999)
	Zymosan	Fungi	Ozinsky et al. (2000)
TLR7	Imidazoquinoline	Synthetic compound	Hemmi et al. (2002)
	Loxoribine	Synthetic compound	Heil et al. (2003)
	Bropirimine	Synthetic compound	Heil et al. (2003)
	Single-stranded RNA	Viruses	Heil et al. (2004), Diebold et al. (2004)
TLR8	Imidazoquinoline	Synthetic compound	Jurk et al. (2002)
	Single-stranded RNA	Viruses	Heil et al. (2004)
TLR9	Unmethylated CpG DNA	Bacteria and virus	Hemmi et al. (2000)
TLR10	Unknown	Unknown	
TLR11	Unknown	Uropathogenic bacteria	Zhang et al. (2004)
		Profilin-like protein from *Toxoplasma gondii*	Yarovinsky and Sher (2006)
TLR12	Unknown	Unknown	Mishra et al. (2008)
TLR13	Unknown	Unknown	Mishra et al. (2008)

Adapted from Akira and Takeda (2004).

Elazar et al. (2010a) showed that alveolar macrophages are both sufficient and essential for neutrophil recruitment elicited by LPS/TLR4 signalling in the mammary gland. Mice depleted of mammary alveolar macrophages were completely refractory to an intramammary challenge with LPS. The authors suggested that macrophage produced TNF-α is the essential signal for the immune cascade resulting in neutrophil recruitment to the mammary gland. Mammary alveolar macrophage depleted mice infected with live *E. coli* bacteria resulted in *E. coli* invaded mammary alveolar epithelial cells with intracellular bacterial communities (Elazar et al., 2010b). Despite the importance of LPS/TLR4 signalling in gram-negative infections, Gonen et al. (2007) described that intramammary infection of mice with *E. coli* P4 resulted in inflammation even in the absence of LPS/TLR4 signalling. Similarly, Elazar et al. (2010b) showed that mice depleted with mammary alveolar macrophages recruited neutrophils normally after a challenge with live *E. coli* bacteria. This inflammatory response indicated that additional factors (ligands) beyond LPS and additional cells beyond alveolar macrophages play a role in the inflammatory response to intact *E. coli* bacteria (see below). However, in the absence of functional TLR4 the infecting *E. coli* P4 invaded the epithelial cells with high efficiency, forming intracellular micro-colonies. The epithelial cell invasion by *E. coli* P4 was not observed in the wild type mice (Elazar et al., 2010b). These results indicate that *E. coli* had a mammary epithelial invasive potential, which is limited by alveolar macrophages using a process dependent on TLR4 signalling. The biological mechanism that allows TLR4 signalling to prevent epithelial invasion is currently not known. Epithelial invasion is one of the distinctive

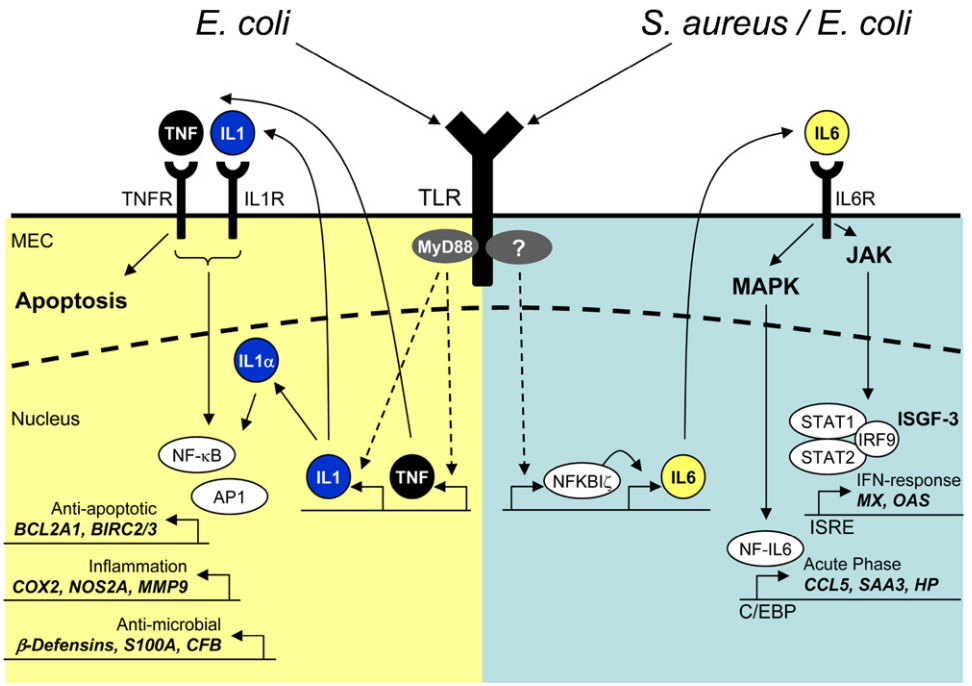

Fig. 1. Pathogen-specific activation of the immune response in MEC. *E. coli* activates the expression of the three master cytokine IL-1, TNF-α and IL-6. *S. aureus* only drives significant IL-6 expression via a MyD88 independent signal transduction. This depends on strong induction of the unusual NF-κB factor NF-kBζ.
Adapted from Günther et al. (2011).

features of persistent intramammary *E. coli* infections (Döpfer et al., 2001; Dogan et al., 2006; White et al., 2010).

The basic structure of LPS in all gram-negative bacteria is composed of lipid A, the inner and outer core region and the O-polysaccharide chain (Brandenburg and Wiese, 2004). The biological activity of the highly hydrophobic and toxic lipid A appears to depend on a peculiar, pathogen-related conformation with lipid A of *E. coli* having the highest toxicity (Caroff and Karibian, 2003). A tailored inflammatory host response should guarantee pathogen elimination. LPS is considered as the main pathogen component initiating inflammation after host pathogen contact via the TLR4 pathway in mastitis caused by gram-negative bacteria. Although other TLR ligands of gram-negative bacteria [CpG-DNA, Flagellin or Curli (Karczmarczyk et al., 2008)] are likely involved in the triggering of the host response, their impact on the host pathogen interaction during coliform mastitis has not been investigated. TLR-ligand induced mechanisms in general do not seem to be highly pathogen specific, although the clinical course of infection and the host innate responses may vary between gram-negative mastitis pathogens (Bannerman et al., 2008). It is hypothesized that such variations may rely on subtle differences in the molecular structure of their PAMPs.

More recently, Sander et al. (2011) showed a distinction in immune response to viable and dead *E. coli* in murine macrophages. Viable *E. coli* bacteria elicited a much more aggressive immune response characterized by higher IFN-β and IL-1β secretion compared to heat killed bacteria or LPS. The authors then showed that so-called viability associated PAMPs (vita-PAMPs) were responsible for the increased immune responsiveness, and prokaryotic MRNA was shown to be one of the responsible vita-PAMPs (Sander et al., 2011). These findings would indicate that the immune system actively gauges the infectivity of the invading pathogens, and responds accordingly.

3.1.2. Cytokine profiles

3.1.2.1. Pro-inflammatory cytokines. Some cytokines clearly promote inflammation. The most important pro-inflammatory cytokines are tumour necrosis factor (TNF)-α and interleukin (IL)-1 which may be subdivided into the cytoplasmatic IL-1β and the secreted IL-1β. TNF-α and IL-1β are key mediators of both, the local and the systemic immune response. They regulate not only the expression of an orchestra of immune response genes including other cytokines, enzymes for the eicosanoid synthesis, and acute phase proteins, but also genes involved in cell proliferation and apoptosis. IL-6 is a key pro-inflammatory cytokine which has also anti-inflammatory properties. Cows infected with different species of mastitis pathogens respond with a strong rise in the abundance of mRNAs encoding these cytokines in the udder tissue as well as increasing cytokine abundance in milk (Buitenhuis et al., 2011; Bannerman, 2009; Kauf et al., 2007; Yang et al., 2008; Günther et al., 2010). Infection with gram-negative bacteria expressing the endotoxin LPS on their surface, like *E. coli*, provoke a much stronger increase in TNF-α and IL-1β expression and secretion than gram-positive pathogens. Infections with *S. aureus* are characterized by a relatively low and short duration increase in pro-inflammatory cytokines, with an important role for

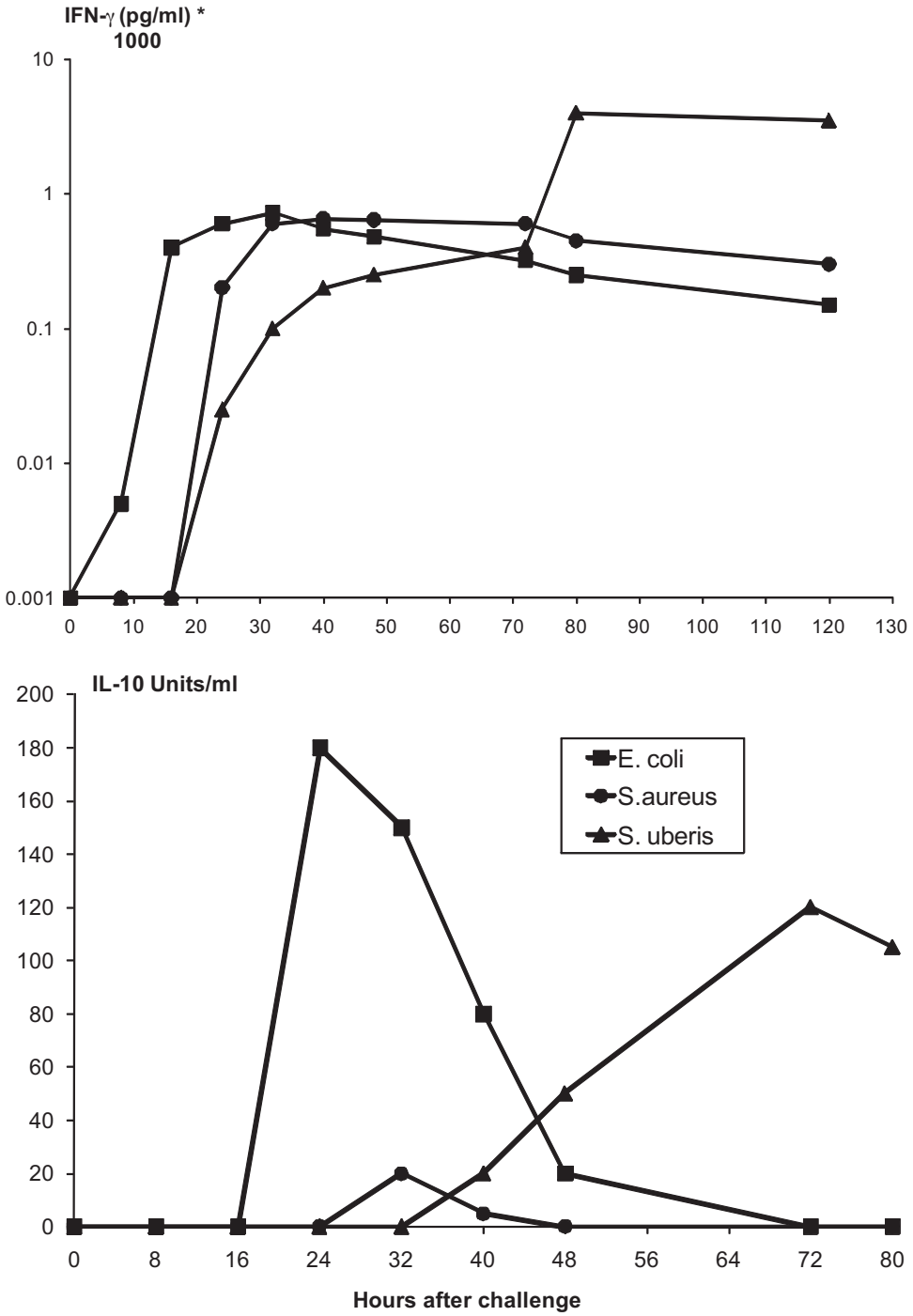

Fig. 2. Cytokine profiles for IFN-γ (top) and IL-10 (bottom) are shown for challenge infections with *E. coli*, *S. uberis* and *S. aureus*. Adapted from Bannerman (2009).

IL-6. Infections with *S. uberis* show a pro-inflammatory cytokine response pattern with an initial delayed response but with ultimately a very strong expression of these cytokines (Bannerman, 2009). LPS mediates a strong pro-inflammatory reaction and this conceivably causes acute mastitis after infection with those gram-negative pathogens. Cytokine profiles of a challenge infection with *E. coli*, *S. uberis* and *S. aureus* are shown in Fig. 2.

Mammary epithelial cells surround alveoli in the milk parenchyma in the udder. They constitute a dominant cell type of the udder parenchyma and have recently been extensively analysed regarding the expression and

secretion of pro-inflammatory cytokines (Griesbeck-Zilch et al., 2008; Günther et al., 2009, 2010; Lahouassa et al., 2007; Yang et al., 2008). The MECs are an important source of these signalling molecules in the udder. Myeloid leucocytes are also able to express TNF-α, IL-1β, and IL-6 in response to an inflammatory stimulus but these cells gain their relevance as sources for newly synthesized and secreted cytokine only later during infection.

3.1.2.2. Anti-inflammatory cytokines. Cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)-β suppress the production of inflammatory cytokines. These interleukins originate from leucocytes invading the udder during infection. One of the best examined anti-inflammatory interleukins in udder immune response is IL-10. This interleukin has two major functions, the inhibition of cytokine synthesis and the reduction of factors of the MHC-II complex. However, IL-10 is also involved in uptake of antigens and differentiation and function of T and B lymphocytes. A significant increase in the amount of IL-10 in milk after infection with *E. coli* was observed, while *S. uberis* infection again showed a delayed response (Bannerman, 2009). However, an infection with *S. aureus* does not induce a significant IL-10 response in the udder (Bannerman et al., 2004b). These patterns are shown in Fig. 2.

TGF-β is another well known anti-inflammatory cytokine. The various TGF-β isotypes share in common many biological activities. Beside their immune suppressive properties they have well described effects on cell proliferation and differentiation. TGF-β1, -β2, and -β3 are expressed in udder tissue (Maier et al., 1991). However, only TGF-β1 and -β2 could be found in milk. Udder infection increases their abundance (Chockalingam et al., 2005; Ginjala and Pakkanen, 1998; Pakkanen, 1998).

Interferons (IFNs) form a cytokine family with immune modulatory functions. Type I interferons are expressed by all cell types but IFN-γ the most extensively studied interferon in udder immunity, is produced exclusively by leucocytes. This cytokine is known to induce macrophage functions such as antigen presentation and increasing lysosome activity. Further, IFN-γ promotes Th1 differentiation and concomitantly suppresses the activity of Th2 cells. Increasing abundance of IFN-γmRNAs was detected in milk cells as well as increasing protein amounts in milk isolated in the course of mastitis (Bannerman, 2009; Kauf et al., 2007; Lee et al., 2006; Riollet et al., 2001). Interestingly relatively high concentrations of IFN-γ could be detected in the milk of animals infected with pathogens causing persistent infections at relatively late time points (>72 h after challenge) (Bannerman et al., 2004b,a; Kauf et al., 2007). This was discussed as an indication of a high cell mediated immune response in the later stages of infection balancing the failure in pathogen clearance caused by an ineffective induction of pro-inflammatory immune reaction.

3.1.2.3. Chemokines. Chemokines are a specific class of cytokines mediating the recruitment of immune effector cells to the site of inflammation. While recruited myeloid- (granulocytes, monocytes, macrophages) and NK-cells ultimately mediate pathogen clearance, immigrating T- and B-cells enable the induction of a humoral immune response in the udder. Examination of the MEC-transcriptome after an inflammatory challenge demonstrated that this cell type is capable to express most of the chemokines with relevance for the immune response in the udder, with the notable exception of the macrophage inflammatory proteins MIP-1α (CCL3) and MIP-1β (CCL4) (Günther et al., 2009). Both the latter chemokines are known to be expressed by myeloid cells which have previously been stimulated with bacterial endotoxins. Hence, MECs play a key role in immune cell recruitment at the early stages of mastitis since they represent the largest cell population in the healthy udder and most likely set in motion the first response to defend the invading pathogen.

CXCL8 (IL-8) is the best examined chemokine in udder immune response. Many studies demonstrate a significant increase in the CXCL8 mRNA abundance in udder tissue and of the CXCL8 protein in milk after infection with various mastitis pathogens (Bannerman, 2009). This chemokine interacts with CXC chemokine receptor 1 (CXCR1) and CXCR2 mediating the recruitment of granulocytes and the accumulation of macrophages to the site of infection.

Neutrophil granulocytes constitute a first line of cellular immune defence and are recruited by CXCL8 early after infection (Riollet et al., 2000). It was found that the CXCL8-encoding gene is one of the first genes revealing induced expression in MEC after an inflammatory stimulus. Other chemokines which are capable to interact with CXCR2 have not so extensively been examined in mastitis research (Rainard et al., 2008). However, transcriptome and RT-PCR analyses revealed that the concentrations of mRNAs encoding CXCL2 (alias CXCL1), CXCL3, and CXCL5 (alias CXCL6) are strongly increased in udder and cultured MEC after inflammatory stimulation (Günther et al., 2009, 2010, 2011; Lutzow et al., 2008).

Monocytes/macrophages constitute the second line of cellular immune defence. They are relevant for innate immunity but also help initiating the humoral immune defence. CCL2 (alias MCP-1) is known as a key chemokine for monocyte recruitment. Yet, only few studies demonstrated an inflammation induced increase of CCL2 mRNA in udder and MEC (Lutzow et al., 2008; Mount et al., 2009; Strandberg et al., 2005).

CCL5/RANTES is strongly expressed in the infected udder as well as in inflammatory stimulated MEC (Griesbeck-Zilch et al., 2008; Mount et al., 2009; Pareek et al., 2005; Günther et al., 2010). This chemokine is known to induce eosinophil recruitment through binding to the CCR3 receptor, which is highly expressed on these cells. The interaction of CCL5 with CCR1 and CCR5 is involved in the trafficking of T-cells and monocytes to the sites of inflammation.

Transcriptome analyses of cytokine expression induced by *E. coli* mediated immune response in udder and cultured MEC revealed that the mRNA of CCL20 is very strong increased in both, udder and MEC (Günther et al., 2009, 2010). CCL20 is the only chemokine known to interact with the receptor CCR6 (Schutyser et al., 2003), a property shared with the antimicrobial β-defensins also known to be strongly increased in the course of mastitis. The ligand–receptor pair CCL20–CCR6 is responsible for the chemo-attraction of immature dendritic cells,

effector/memory T-cells, and B-cells. The recruitment of this cell types provide a link to the humoral immune response.

The analysis of global changes in gene expression by microarray analysis revealed that the expression of more cytokines than those discussed above is regulated in the infected udder (Günther et al., 2009, 2010; Lutzow et al., 2008). These studies altogether show that *S. aureus* induces the expression of these factors to a significantly lower degree than *E. coli*. A more extensive analysis of the orchestrated interaction of all these cytokines/chemokines during mastitis will help to better understand the regulation of the pathogen specific immune response in the udder and eventually the etio-pathology of mastitis.

3.1.3. Cellular immune response

Milk in healthy cows has a resident population of immune cells. This population is generally dominated by macrophages but also contains neutrophils and lymphocytes (Sordillo, 2005). In addition, the ability to recruit cells into the mammary gland during the bacterial growth phase is considered pivotal since a 1 h delay in recruiting PMN can result in an 8-fold increase of *E. coli* (Hill, 1981) as shown in Fig. 3. The faster the host can recruit effector cells, the earlier bacterial growth will be decelerated. Of significance here is that cows with very low somatic cell counts (SCC) in milk (Wellnitz et al., 2010) and herds with low bulk milk SCC (Schukken et al., 1989) showed to be of higher risk for more severe gram-negative IMI. Normal counts of immune cells in healthy mammary quarters range between 20,000 and 100,000 cells/ml (Green et al., 2006; Schepers et al., 1997). Mammary quarters with lower cell counts tend to response less efficient to an intramammary challenge (Wellnitz et al., 2010) and also show higher incidences of clinical mastitis (Peeler et al., 2003).

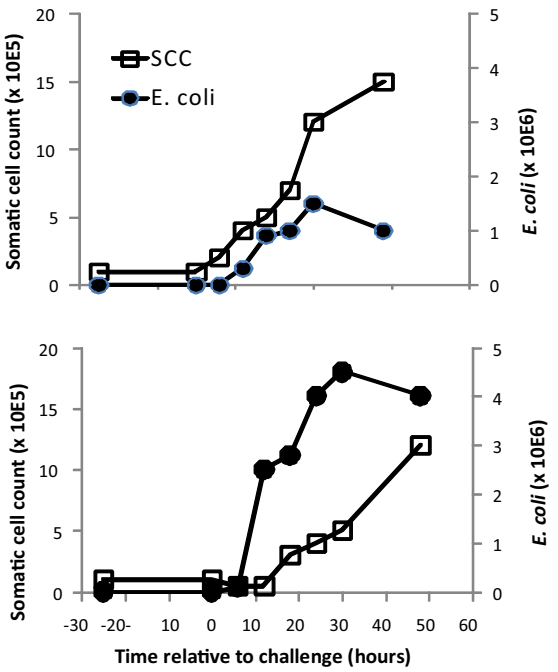

Fig. 3. Growth of *E. coli* ($\times 10^6$/ml; ●) in inoculated mammary glands and pattern of leukocyte influx measured as somatic cell count ($\times 10^5$/ml; □) into milk in moderate responders (top) compared with severe responders (bottom) during experimentally induced *E. coli* mastitis. Adapted from Burvenich et al. (2003).

Recent studies elucidated the roles of macrophages and neutrophils in the response to an *E. coli* IMI. Elazar et al. (2010a,b) showed that mice depleted of mammary alveolar macrophages were not able to mount an immune response to LPS. Even though the alveolar macrophage depleted mice did mount a neutrophils response to an intramammary challenge with live *E. coli*, these bacteria invaded the mammary alveolar epithelial cells and formed persistent intracellular bacterial communities. Infection of mice that were depleted of neutrophils prior to IMI showed unrestricted bacterial growth, tissue damage, severe sepsis and mortality. This study suggests that neutrophils provide an essential antimicrobial defence against an IMI with *E. coli*. Whereas alveolar macrophages play a crucial role in LPS/TLR4 signalling and in the prevention of *E. coli* invasion and establishment of intracellular bacterial communities in the mammary epithelium.

Lymphocytes are divided into 2 main groups: T and B lymphocytes. The T lymphocytes can be classified further into αβ T lymphocytes, which include CD4+ (T helper) and CD8+ (T cytotoxic) lymphocytes, and γδ T cells. In the lactating mammary glands, αβ T lymphocytes prevail and predominantly express the CD8+ phenotype (Shafer-Weaver et al., 1996). The function of activated cytotoxic T cells (CD8+) is to kill host cells infected with a pathogen, as detected by antigens expressed on the surface of infected cells. Helper T cells (CD4+) have a more indirect but equally important effect on the infection. When a T_H cell matures, it develops into 1 of 4 types of T_H cells. Stimulation of these mature T_H cells can cause the expression a large variety of cytokines that can direct the immune response toward a pro-inflammatory cytotoxic T cell-mediated (T_H1), B-cell-mediated (T_H2), neutrophil-mediated response (T_H17), or to counter-regulate the response (T_{reg}). During bacterial infection of the bovine mammary gland, large numbers of leukocytes migrate into the udder, resulting in the establishment of a host response against the pathogen. Currently, the specific leukocyte populations mediating this immune response are not well defined. Cell surface markers are used to identify the specific cell populations identified in the mammary immune response. There is an increasing range of well-characterized monoclonal antibodies (mAbs) available for, and raised against, bovine cell surface markers. A list of bovine specific antibodies against cell surface markers is maintained by the US veterinary immune reagent network and is accessible at http://www.umass.edu/vetimm/ruminants/index.html.

Descriptive studies in healthy cows have been done to describe the normal cell population in blood and milk. Park et al. (1992) showed marked changes in milk T lymphocytes proportion during the lactation cycle as shown in Fig. 4. Blood and milk from healthy cows and cows with naturally occurring mastitis have been evaluated to determine if distinct αβ ($CD2^+$) and γδ ($CD2^-$, $WC1^+$) T-lymphocyte subsets were involved in the response of the udder to a mastitis pathogen and if the type of mastitis pathogen influenced the subset composition of these

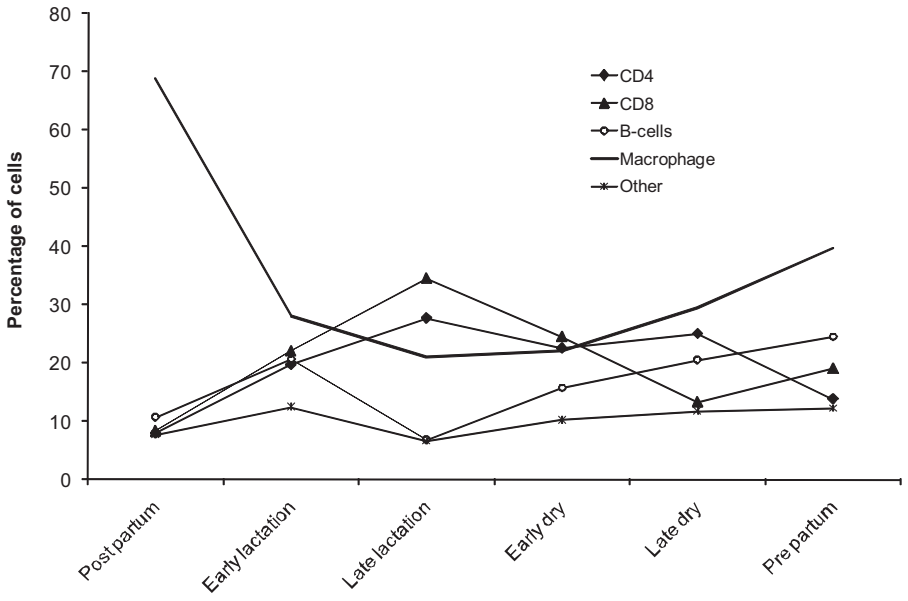

Fig. 4. Percentage of milk lymphocytes in healthy animals during a full lactation cycle. Adapted from Park et al. (1992).

responding leukocytes. Typically, blood samples from cows with confirmed staphylococcal and streptococcal mastitis were characterized by increased numbers of γδ T cells, the most dramatic changes in leukocyte distributions occurred in milk samples from cows with intramammary infections. It was observed that there was a 75% increase in αβ T-cell levels and a 100% increase in γδ T-cell levels relative to the levels in milk samples from healthy animals. The increase in αβ T-cell numbers observed in milk from cows with staphylococcal mastitis was primarily due to increased numbers of CD4+ T cells, while the increase in αβ T-cell numbers observed in cows with streptococcal mastitis was due to a parallel increase in both CD4+ and CD8+ T-cell numbers (Sordillo, 2005). It was also found that, in comparison to the situation for healthy cows, L-selectin was down-regulated and CD18 was up-regulated on leukocytes from cows with mastitis (Sordillo, 2005). These studies suggest that distinct αβ and γδ T-cell subsets are involved in the host defence of the udder against intramammary infection and that selective recruitment of these T-cell subsets depends on the infectious agent involved (Soltys and Quinn, 1999). Shafer-Weaver et al. (1999), studied CD4(+) T_H-1 and T_H-2 sub-populations in the post partum cow. The CD4(+) cells isolated postpartum had enhanced interleukin-4 and interleukin-10 mRNA transcript expression. Their observations suggest that CD4+ lymphocytes act predominantly towards a T_H-2 rather than T_H-1 bias in the peri-parturient physiological state of the dairy cow. It may therefore be suggested that T_H-2 bias and the associated repertoire of cytokines produced, may be an underlying reason for diminished host immune response during the prepartum and immediate postpartum period. A very similar finding was reported by Asai et al. (1998) who evaluated the CD4/CD8 ratio during lactation and the dry period. The early lactation CD4/CD8 ratio was approximately .5, it increased to .8 in late lactation and further increased to values between 2.5 and 4 in the dry period. Asai et al. (1998) also observed that the predominant cytokines produced by the CD4+ cells in the dry period were IL-2 and IL-4.

Mehrzad et al. (2008) studied the T-cell dynamics after an experimental *E. coli* challenge infection. The absolute and relative amount of CD4+, CD8+ and CD21+ T-lymphocytes were studied in blood and milk. The cows were inoculated intramammary with *E. coli*. The CD8+ cells were the first-recruited T cells in the milk. There was a significant decline in the CD4+/CD8+ ratio at 6–24 h after challenge due to greater CD8+ cell concentrations in milk. At 72 h post challenge the CD4+/CD8+ ratios increased about 2-fold due to an increase in CD4+ cell concentration. The increased concentration of CD4+ cells at 72 h post challenge coincided with an increase in the CD21+ cell population in the milk. This study demonstrated that after *E. coli* challenge a dynamic influx of CD8+ followed by CD4+ lymphocytes occurs. It is unknown whether such lymphocyte dynamics are bacterial pathogen specific or whether this is a generic inflammatory patterns across pathogens. Given the large difference in pathogen specific response in cytokines, it may be expected that pathogen specific T-cell dynamics are present. Similarly, it may be expected that stage of lactation would also affect the pattern of cellular response to an IMI (Shafer-Weaver, 1999). Further work in cellular immune response to bacterial infections in the mammary gland is clearly warranted.

Mammary epithelial cells have shown to be highly immune competent (Strandberg et al., 2005; Lahouassa et al., 2007; Yang et al., 2006; Günther et al., 2009). Pathogen contact induces in these cells a burst of cytokine and chemokine synthesis which is much stronger after a challenge with *E. coli* than it is elicited by *S. aureus* (Yang et al., 2008; Günther et al., 2010). In the healthy gland,

MEC outnumber by far any other cell type likely to coming into contact with an invading pathogen. Thus, quality and extent of the pathogen dependent activation of the immune functions of these cells is likely involved in eradication of the pathogen or manifestation of the infection.

Pathogen-dependent induction of immune functions in MEC reveals several cell type and gene specific features. TLR activation results in many cell types in the activation of NF-κB transcription factors, as already mentioned. These factors are known master regulators of inflammation controlling the expression of >100 immune relevant genes (Ghosh and Hayden, 2008; Pahl, 1999). However, this does not occur in MEC, if heat inactivated *S. aureus* particles are the pathogen stimulus (see above). Yet, the very same pathogen preparations will activate NF-κB in RAW264 macrophages. Moreover, the NF-κB p65 factor is known to drive the expression of many genes. However, while activation of this factor is crucial for the expression of β-defensins in MEC, elevated levels of this factor alone is not sufficient to stimulate the very same genes in HEK293 cells (Yang et al., 2006). Moreover, while NF-κB p65 has been identified in many different cell types as the key driver for induced IL-8 expression, it was recently found that in MEC its function is to down-regulate and confine pathogen-induced IL-8 expression (Liu et al., 2011).

Induction of immune functions in MEC depends on the pathogen species. *E. coli* activates the expression of the full repertoire of genes encoding both, sentinel as well as effector genes of immune function. This stimulus increases within 24 h the mRNA concentrations up to 1000-fold for key cytokines (TNF-α, IL-1, IL-8) and ∼100-fold for several effector genes of immune defences (β-defensins, NOS2A, SAA3) (Yang et al., 2006; Günther et al., 2009). This is accomplished via the activation of the relevant TLR-receptors and their downstream signalling cascade (Fig. 1). Induction of these genes by *S. aureus* is diminished (Lara-Zárate et al., 2011). This is in part due to an impairment of MyD88 signalling, immediately downstream from the trans-membrane TLR receptors (e.g. TLR2, TLR4). *S. aureus* apparently prevents the formation of the so called Myddosome around the TIR domain of the TLR receptors forming the structural platform for the attachment of further downstream acting factors (Lin et al., 2010; Motshwene et al., 2009). This points to events occurring immediately underneath the membrane of the host-cell as the area, where *S. aureus* intervenes with TLR-mediated signal transduction in MEC (Fig. 1). Structural proximity of these events to the apical membrane of the MEC is also suggested by the fact, that inactivated (either by heat or UV-radiation) *S. aureus* particles are incapable to actively invade the host cell. Hence, their contact with the membrane of the host cell triggers impairment of auxiliary mechanisms of TLR-signalling which are mandatory for recruiting MyD88 to the TIR domain of the receptor (Kagan and Medzhitov, 2006). As a consequence, *S. aureus* elicits an immune reaction in these cells solely dominated by the IL-6 cytokine, while *E. coli* also activates two additional cytokines, IL-1 and TNF-α (Fig. 1). Consequently, the positive feed back loop of cytokine production known to be induced by IL-1 and TNF-α is diminished in the *S. aureus* triggered immune response of the MEC.

3.2. Adaptive immune response

The adaptive immune system can not only specifically recognize a species of microbe, but also distinguish variants of a species. Antibodies generated by B cells recognize whole antigens, whereas the T-cell receptors recognize fragments of antigens presented by specialized molecules called major histo-compatibility complex (MHC) class I or class II molecules.

3.2.1. Antibody based response

3.2.1.1. E. coli. The adaptive immune response to IMI has mostly been studied in relationship to either *E. coli* or *S. aureus* IMI. Commercial vaccines are available for both these organisms, although the efficacy of the vaccines to protect against IMI with these two organisms is still debated.

Vaccination with a core J5 *E. coli* vaccine is commonly practiced on dairy farms in the USA and commercial J5 vaccines are now also available in Europe. The J5 vaccine is assumed to be effective in reducing severity of clinical mastitis (González et al., 1989). Higher J5-specific IgG1 and IgG2 antibody are typically observed in J5 vaccinates after vaccination. A distinguishing feature of immunological memory is the irreversible B cell genetic change from IgM production to production of other antibody isotypes, including IgG1 and IgG2 (Burton et al., 2003; Estes et al., 1998). In the bovine as well as in several other species, an immune response with more production of IgG2 antibody has been recognized as part of a Th1 or pro-inflammatory response, while a response with more IgG1 is part of a Th2 or anti-inflammatory response (Stevens et al., 1988). Because IgG2 is an important opsonizing antibody aiding in neutrophil phagocytosis of bacteria, and IgG2 has the ability to readily fix complement, it has been suggested that an IgG2 Th1-type response might be beneficial against bovine mastitis (Bastida-Corcuera et al., 1999; Burton et al., 2003; Dosogne et al., 2002). Stage of lactation affects this polarized immune response in lactating cows; the immune response in late gestation presumably has a Th2 bias, while in lactation the bias is toward a Th1 response, which is generally considered to be associated with greater protection against mastitis (Shafer-Weaver et al., 1999). Antibody responses of Holstein dairy cattle vary between animals and have been categorized in populations of animals as either high, average, or low following vaccination with ovalbumin and J5. Animals in the high serum antibody response group had the lowest rate of CM overall, and those in the low serum antibody response group had the highest rate of CM overall (Wagter et al., 2000).

There is some evidence that J5 vaccination may also induce cell-mediated immunity. Live *E. coli* J5 bacteria were reported to stimulate approximately 200 human PMN per bacteria in vitro, and stimulate PMN much more efficiently than LPS or other strains of *E. coli* did. The mechanism for the PMN stimulation was not identified, and no comparison was made with J5 immunization (Katz et al., 1996). Possible cell-mediated immune response(s) against coliform bacteria which may be stimulated or enhanced by J5 vaccination is an area in need of further study (Dosogne et al., 2002).

Wilson et al. (2007, 2008) provided evidence that increased production of both J5-specific IgG1 and IgG2 antibodies are important mechanisms of J5 vaccine protection, including production of a higher proportion of IgG2 than in non-vaccinates, a Th1 biased response. Serum ratio of J5-specific IgG1:IgG2 was reported to be less than one in vaccinates post-calving, thus demonstrating a Th1 biased response after calving. It was previously found that phagocytosis by PMN and the associated clearance of coliform bacteria from the mammary gland was optimal when IgG2 increased within 4 h following IMI, 6–12 h before the greatest influx of PMN from blood to milk (Burton et al., 2003; Rainard, 1983). It may then be hypothesized that J5 vaccination is associated with reversing the natural trend in early lactation cows toward a less protective Th2 response; instead J5 vaccinates appear to show a more beneficial Th1 response (Wilson et al., 2008).

Cows that are vaccinated with J5 characteristically show a proportionally lower IgM antibody response and a relative increased IgG1 or IgG2 response after an *E. coli* IMI. Several studies have shown that an IgM dominated response in cattle may be less successful than a response with more class switching to IgG1 or IgG2 antibodies against J5 (Burton et al., 2003; Dosogne et al., 2002).

Immunization with J5 against gram-negative bacterial infections and the importance of an increased IgG concentration in serum as a potential protective mechanism has been reported in other species. An experimental vaccine against gram-negative bacterial sepsis in humans, complexes J5 LPS with outer membrane protein of *Neisseria meningitides* bacteria. Rats and mice vaccinated (2 or 3 doses) with the J5-*Neisseria* bacterin were then inoculated with *Pseudomonas* or *Klebsiella* orally, intra-peritoneally, or into cecal ligation to mimic bacterial sepsis. After challenge, vaccinates had significantly increased (approximately 7-fold higher) survival in association with increased IgG antibody specific for J5 LPS compared with controls (Cross et al., 2001; Opal et al., 2005).

Immunological memory stimulated by J5 vaccination is generally associated with lower bacterial growth after IMI, a reduced milk production loss and lower cull rates following clinical mastitis compared to unvaccinated controls. These benefits decrease on a continuous basis as lactation progresses, a waning of vaccine protection over time. This raises the question of the optimum J5 immunization schedule for producing long-lasting immunological memory associated with sero-conversion to long-lasting high titers of anti-J5 antibody. Based on a study in steers, the authors suggested that a large number of doses of J5 bacterin may be needed to result in high concentration of IgG2 reactive against J5 (Chaiyotwittayakun et al., 2004).

3.2.1.2. S. uberis. Major efforts have been invested towards designing a vaccine against *S. uberis*. The activation of immunity against *S. uberis* mastitis was initially demonstrated by Hill (1988), who infected cattle with *S. uberis* via the teat canal and found that following cure of the initial infection a significant proportion of the animals were refractory to subsequent infection. Hill (1988) suggested that an observed T-cell increase might be considered responsible for the observed protection. Subsequent studies using crude vaccines have shown that heat-killed *S. uberis* administered locally to the mammary gland could increase resistance against homologous challenge, i.e. the same strain as the vaccine (Finch et al., 1997). Attempts at protection via a humoral response against *S. uberis* have consisted of vaccinating cows with a cloned Gap C molecule (Fontaine et al., 2002), plasminogen activator, PauA (Leigh et al., 1999) and with a *S. uberis* adhesion molecule (Almeida et al., 2006). Vaccination typically induced high levels of antibodies in mammary gland secretions but the impact of these antibodies on protection against IMI and clinical mastitis has not been convincingly shown. In the case of Gap C, vaccination did result in less severe inflammatory response (Fontaine et al., 2002). In the case of PauA vaccination protected against IMI with *S. uberis* that possess this gene, but *S. uberis* isolates with alternative pathways have been shown to exist on commercial dairies. A significant obstacle in the design of an effective vaccine against *S. uberis* is the high level of genetic variability. This large variation will make the selection of vaccine antigen candidates more difficult. The distinction between antibody based or cellular based memory may be of particular importance in designing vaccines for *S. uberis* IMI.

3.2.1.3. S. aureus. A number of studies have been published on antibody driven vaccination to prevent staphylococcal (predominantly *S. aureus*) IMI. Leitner et al. (2003) described a field study of a *S. aureus* vaccine. A total of 452 Holstein heifers were included in the trial with 228 heifers being vaccinated and 224 serving as unvaccinated controls. Antibody response was detected in all vaccinated animals 4–5 weeks post-primary immunization and it was sustained for approximately 300 days. No significant difference in *S. aureus* infections was observed, in the vaccinated group 1.3% of heifers became infected and this was 2.7% in the control group.

Middleton et al. (2006) performed a challenge study in vaccinated and control heifers. All heifers were challenged with a heterologous strain of *S. aureus* by intramammary infusion on days 6–8 of lactation in a single infection-free mammary quarter. All cattle became infected with *S. aureus* after challenge and there were no differences in *S. aureus* clearance rates between groups. Vaccinated heifers did show a lower mean duration of clinical mastitis and lower total mastitis score post-challenge than controls. Nickerson et al. (1999) vaccinated heifers at 6 months of age followed by a booster dose 2 weeks later and subsequent vaccinations every 6 months until calving. Vaccinates had a significant reduction in both new *S. aureus* IMI during pregnancy and new *S. aureus* IMI at calving relative to controls (Nickerson et al., 1999). More recently, Prenafeta et al. (2010) evaluated a *S. aureus* vaccine based on an extracellular slime associated antigenic complex from *S. aureus*. Twelve animals were vaccinated at 45 days before the expected parturition date and revaccinated 35 days later. All cows were challenged with a heterologous strain of *S. aureus* 23 days after calving. Immunization enhanced antibody titers against the slime associated complex. However, there was no evidence of a difference between vaccinated and control groups with regard to IMI and clinical signs of mastitis following the challenge. Vaccinated cows showed

a reduced *S. aureus* concentration in milk during the post-challenge period.

The results of these vaccination studies indicate that the vaccination typically results in an increase in antibody titers but not in a complete prevention of *S. aureus* IMI (Tuchscherr et al., 2008). The data would suggest that vaccination may be associated with lower bacteria counts in infected animals.

3.2.2. Endotoxin tolerance

Repeated exposure towards bacteria or PAMPs can lead to a subsequent refractory state in mammals resulting in a decreased cytokine production such as TNF-α and IL-1β (Mages et al., 2007). This phenomenon is known as endotoxin tolerance (ET) and has been widely investigated in the human and murine system. ET can be regarded as a potentially protective mechanism against septic shock by preventing an excessive overshoot of systemically harmful factors. It was found in murine macrophages that epigenetic control mechanism involving chromatin compaction prevent excessive induction of cytokine encoding genes during repeated LPS stimulation. Cattle may show a decreased immune response to repeated LPS exposure. In bovine mastitis models it was shown that subsequent intramammary challenge with *E. coli* in an interval of 14 days was accompanied by a reduction of the acute phase response and clinical signs (Suojala et al., 2008). The potentially protective effect appears to be of short duration as an interval between clinical cases of more than 14 days did not show any evidence for protection (Schukken et al., 2009). Intramammary infusion with LPS prevented the establishment of IMI 19 h after experimental *E. coli* mastitis (Lohuis et al., 1990). Repeated exposure to LPS showed to have a decreasing impact on the initial drop in milk yield (Shuster and Harmon, 1991). Besides LPS, other TLR ligands can induce ET. In goats it was shown that intramammary infusion with CpG-ODN resulted in a reduced amount of inflammatory cytokines and a shortened duration of disease after subsequent *E. coli* challenge (Zhu et al., 2007). Future research will need to indicate how ET can influence innate immune mechanisms by facilitating a rapid response towards microbial infection.

4. Clinical response patterns

4.1. Clinical signs

Several systems to describe clinical severity of intramammary infections have been proposed. A study by Wenz et al. (2001), proposed a system that was also validated with both clinical observations, bacterial growth and ultimately survival of the animal (Wenz et al., 2006, 2001). This system is based on four classes of clinical symptoms and an evaluation of the milk based on the presence of flakes and clots. The four clinical parameters include rectal temperature, hydration status, rumen contractions and clinical attitude. A score system based on these criteria was developed. The distribution of clinical response patterns for the three bacterial IMI is presented in Table 2. These transient and persistent clinical and subclinical infections were observed in a population of three Dutch dairy farms that were followed intensively during a 2-year period. Clearly, the infection patterns between bacterial species are very different. In the case of *E. coli*, transient (short-term) infections are the most common pattern, although persistent infections with and without clinical flare-ups exist. In stark contrast, the vast majority of IMI of *S. aureus* are persistent subclinical or repeated clinical and only a small number of IMI is transient clinical. Both streptococcal species show a pattern that is somewhat in between these two extremes.

In *E. coli* challenge studies the response of the cow to challenge is often classified as either mild, moderate or severe based on the obtained clinical score. During a mild or moderate intramammary inflammatory reaction, bacterial growth will be under control and only a low number of bacteria will be measured in the mammary quarter. Relative low levels of LPS will be recognized leading to moderate and specific cytokine production by macrophages. These so-called moderate responders typically show a steep increase in SCC after intramammary infection, due to fast migrating neutrophils that inhibit bacterial growth through phagocytosis and killing. In contrast, slow neutrophil influx is observed in severe responders (see Fig. 3). As *E. coli* populations may double every 20 min, a delay of just 20 min in neutrophil arrival in lacteal secretions has a significant effect on the outcome of the infection. For *E. coli* it was shown that the severity of disease correlates strongly with bacterial counts in milk (Hogan and Smith, 2003; Wenz et al., 2006). Hence, a 1-h delay in neutrophil recruitment into the mammary gland could result in an 8-fold larger number of *E. coli* to kill and more endotoxin to detoxify (Burvenich et al., 2003).

The first apparent response to infection with *S. uberis* is an increase in neutrophil numbers in milk. Typically this is detected 24 h following experimental challenge of the lactating bovine mammary gland; considerably later than the neutrophil influx to *E. coli* and other coliform species. At this time the number of neutrophils present in milk is typically between 10^5 and 10^6/ml of milk. During the progression of the infection the number of neutrophils increases; around 10^7/ml milk are typically present at the onset of clinical signs (Hill, 1988; Finch et al., 1997; Bannerman et al., 2004b). The progression of clinical signs of mastitis due to *S. uberis* occurs in both the milk and udder. Initially, clots appear in the milk and ultimately the secretion shows changes to both colour and composition due to the influx of serum products and precipitation of milk proteins.

Clinical response to a *S. aureus* IMI is typically very mild and most often not clinically observable as the majority of IMI result in subclinical infections (Table 2).

4.2. Milk production

Milk production loss due to pathogen specific clinical mastitis was studied in a number of large dairy farms (Gröhn et al., 2004; Schukken et al., 2009). Typically, cows that were going to experience a case of clinical mastitis produced more milk compared to cows that were not affected by clinical mastitis during their full lactation. Milk yield in cows experiencing clinical mastitis generally began to drop 1 or 2 weeks before diagnosis and the greatest loss in milk production occurred immediately following diagnosis. The

Table 2
Clinical signs during infection with a selected number of bovine mammary pathogens. Transient clinical are infection where the bacteria where only isolated at the time of clinical signs, clinical-persistent are clinical cases where the bacteria where first isolated at the time of clinical signs but the infection persisted thereafter, and Persistent clinical were cases where the bacteria where isolated before, during and after the clinical case.

Pathogen	N[a]	Clinical cases (repeats) = total			Subclinical, persistent
		Transient	Clinical-persistent	Persistent	
E. coli	105	89	6 (20) = 26	4 (4) = 8	6
S. aureus	171	21	18 (24) = 42	23 (33) = 56	109
S. uberis	79	28	10 (12) = 22	11 (21) = 32	30

Data are adapted from Lam (1996).
[a] Number of IMI.

most severe drop in milk production was observed in cows with *E. coli* mastitis. It was found in an infection trial that *E. coli* pathogens indeed ablate (e.g. <5%) casein synthesis within 24 h pi. This occurs only in the infected udder quarter (Vanselow et al., 2006). This study showed that epigenetic mechanisms contribute to shutting down the casein synthesis. These involve chromatin compaction at crucial regulatory promoter elements of the αS1-casein promoter associated with infection induced hypermethylation of this regulatory site, insulating the promoter against the systemically prevailing lactation specific high levels of prolactin. Moreover, post translational controls must also be activated preventing translation of the still abundantly present casein mRNAs in the infected udder quarter. Significant milk production loss was also observed for cows with mastitis due to *Streptococcus* spp. and *S. aureus*. However with *Streptococcus* spp. and *S. aureus* cases, milk production loss did not drop as steep as with *E. coli*, but milk loss persisted until at least 70 days after diagnosis. Cows with a case of *S. aureus* mastitis already showed a significant drop in milk production up to 30 days before the actual case, suggestion a subclinical mastitis that developed into a clinical case. This mild reduction of milk synthesis in the case of subclinical persistent *S. aureus* mastitis is conceivably owing to the regionally restricted, patchy reprogramming of individual infected alveoli from milk production to pathogen defence. Such a spatially tightly restricted reprogramming pattern was observed *in situ* hybridizations of serial udder sections from cows suffering from subclinical, persistent *S. aureus* infection after probing for either αS1-casein or β-defensin 5 mRNA molecules (Yang et al., 2006). The milk production patterns of cows with clinical mastitis caused by these three pathogens are shown in Fig. 5.

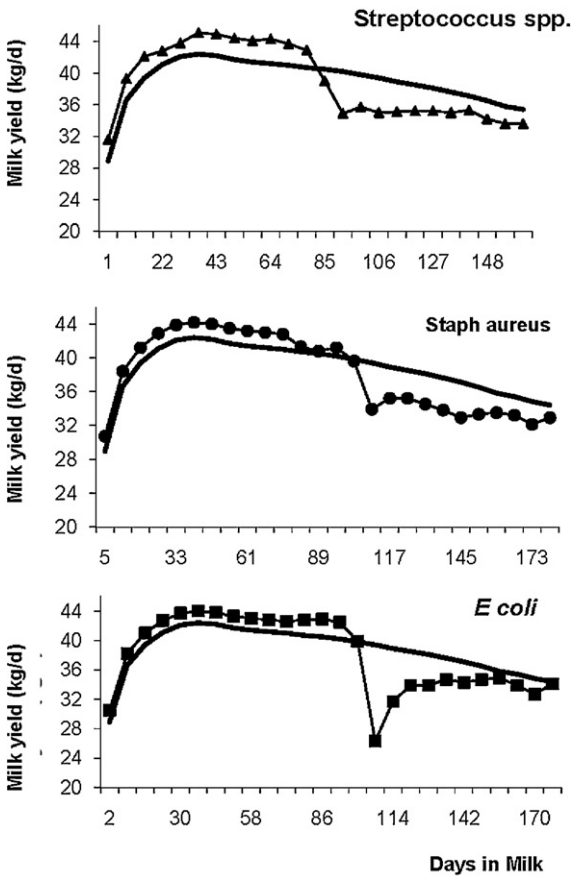

Fig. 5. Lactation curves for multipara cows with no clinical mastitis or one case of either gram-positive or gram-negative clinical mastitis. Adapted from Gröhn et al. (2004) and Schukken et al. (2009).

4.3. Culling

The effects of pathogen specific clinical mastitis on time to culling were studied in 2697 Holstein cows (Gröhn et al., 2005). The overall annual culling percentage was 35.6%. All of the pathogens studied markedly reduced herd life compared to not affected herd mates (Fig. 6). Cows with a clinical case of *S. aureus* had the highest rate of culling with the rate of culling increasing over time since the clinical case. In contrast to this *S. aureus* specific culling pattern, *E. coli* affected cows showed a high rate of culling immediately after the clinical case occurred, but the daily culling rate decreased sharply over time. Cows infected with *Streptococcus* spp. showed a similar culling pattern compared to *E. coli* infected cows, but their initial cull rate was lower compared to the *E. coli* affected animals.

5. Discussion

Although much progress has been made in understanding the pathobiology of mastitis, there are still important areas that remain poorly understood. Among these important gaps in our knowledge are relationship between intramammary infection, TLR-based immune response and the resulting cytokine profiles. The compromised up-regulation of inflammatory cytokines in *S. aureus*

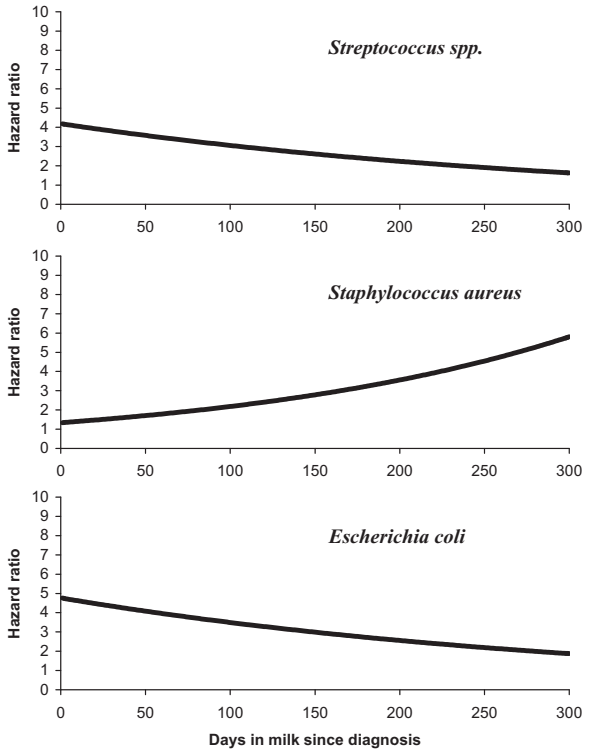

Fig. 6. Hazard ratio of culling of cows with a clinical mastitis case due to *Streptococcus* spp., *S. aureus* or *E. coli* relative to cows without clinical mastitis.
Adapted from Gröhn et al. (2005) and Bar et al. (2008).

infected glands may, at least partially, contribute to the persistent course of infection caused by this pathogen. Further research on identifying factors responsible for the differentially expressed cytokine profiles may be fundamental to developing strategies that mitigate the outcome of bovine mastitis (Mount et al., 2009). There are circumstances where an apparent disconnect exists between IMI and an up-regulation of TLRn and subsequent cytokine production. For example, Yang et al. (2008) reported that infections with *S. aureus* resulted in an up-regulation of TLR2 and TLR4 activity that was similar to *E. coli* but the resulting cytokine profiles proved to be vastly different. The molecular causes for this delay are currently unknown. Once they are unravelled, then these might possibly offer new molecular targets for improved therapy of persistent *S. aureus* infections.

A better understanding of differences in host immune response to different bacterial pathogens may provide opportunities for up or down regulating of the immune responsiveness. The recent finding that epigenetic mechanisms, such as chromatin remodelling are crucial for pathogen-dependent stimulation of β-defensin expression in the udder (Liu et al., 2011) suggests looking at previously unrecognized layers of controls for the synthesis of bactericidal factors in the udder. These may be very significant for pathogen survival early after infection, given the selective enrichment of β-defensin and cathelicidin encoding genes in the bovine genome. This features more than 100 closely related β-defensin-encoding genes (The Bovine Genome Sequencing and Analysis Consortium et al., 2009). 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, it is likely that other cell types (such as mammary epithelial cells) are significant sources of cytokines in the mammary gland and other tissues (Ibeagha-Awemu et al., 2008; Griesbeck-Zilch et al., 2008; Günther et al., 2011).

There is currently a very shallow insight in the cell-mediated immunity as it pertains to mastitis. It is unclear how the cell-mediated immunity cascades after an IMI and whether pathogen specific and lactation stage specific patterns exist. Preliminary evidence would suggest that during late gestation, the cell-mediated immunity is biased toward a T_H2 dominance changing the dominant direction of protection against invading bacteria. Furthermore, pathogen specific importance of the cell-mediated immunity is suspected, with a suggested role for lymphocytes in the acquired protection against *S. uberis* IMI (Hill, 1988). To better understand the pathogenesis of mastitis and increase our ability to modify immune responsiveness, future research into cell-mediated immunity in mastitis is warranted.

Another apparent disconnect between clinical observations and our current understanding of the pathobiology of intramammary infection was observed with *E. coli* IMI in late gestation. These IMI are characterized by the absence of pro-inflammatory cytokines together with the absence of clinical signs of mastitis in the late dry period. Mammary glands are readily infected in this period, but do not show signs of clinical mastitis (Bradley and Green, 2004), whereas immediately after calving clinical mastitis is a disease with a high incidence. It is hypothesized that a Th2 bias is present in late gestation (Saito et al., 2010; Marzi et al., 1996; Wegmann et al., 1993), and this may explain the observed non-occurrence of clinical mastitis signs in late gestation. Hence, a further understanding of the host response and intervention strategies during the dry period is an obvious area for further research.

An ability to modify the immune response to an IMI will likely provide therapeutic opportunities to either up or down-regulate the immune response depending on the clinical condition of the patient. Further genomic and proteomic research on the impact of calving and the start of lactation on transcription of the host genome will provide insight in the underlying reasons for immunosuppression in the peri-parturient period. These findings support a holistic approach to the study of the bovine immune response. These studies would include genetics but also physiological status of the animal. The recent completion and release into the scientific community of the bovine genome (http://www.ncbi.nlm.nih.gov/projects/genome/guide/cow/), provides a unique opportunity to better understand the underlying biological reasons for improved udder health. A recent study linked genetic coding for an impaired cytokine response to an increased susceptibility of subclinical mastitis (Sugimoto et al., 2006).

Such studies will eventually provide us with a directed selection opportunity for improved mastitis resistance.

Only recently, we have started to recognize the potential for a sub-population of host-adapted mammary pathogenic bacteria with an affinity for the mammary gland (Almeida et al., 2011; Bradley and Green, 2001; Dogan et al., 2006; Shpigel et al., 2008). Further research into the characteristics of this sub-population will provide much insight into virulence characteristics of these bacterial pathogens. A better understanding of the pathogenesis of these persistent intramammary infections will provide insight and opportunities to ameliorate the severity of bacterial infections and may eventually lead to better preventative programs and interventions.

Finally, our lack of highly efficacious intervention tools to support the immune response of mastitis affected cows, contributes to pain and suffering in these animals. Further research into the value of immune-altering, symptomatic and antimicrobial therapy is warranted, certainly with regard to newly recognized elements such as pain control and milk production losses. Similarly, prevention of IMI and subsequent clinical mastitis through a next generation of vaccines will provide more long-term solutions to the increasing problem of mastitis in dairy cows.

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