Vet. Res. (2008) 39:05
DOI: 10.1051/vetres:2007044

www.vetres.org

Review article

The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of *Escherichia coli* mastitis in dairy cattle

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(Received 5 October 2006; accepted 2 August 2007)

Abstract – Mastitis is one of the most costly production diseases in the dairy industry that is caused by a wide array of microorganisms. In this review, we focus on the Gram-negative *Escherichia coli* infections that often occur at periods when the innate immune defence mechanisms are impaired (i.e., parturition through the first 60 days of lactation). There is substantial evidence demonstrating that at these periods, the expected influx of polymorphonuclear neutrophil leukocytes (PMN) into the mammary gland is delayed during inflammation after intramammary infection with *E. coli*. Here, we provide some hypotheses on the potential mechanisms of action on how the disease may develop under circumstances of immunosuppression, and describe the potential involvement of the toll-like receptor-4 signal transduction pathway in the pathogenesis of *E. coli* mastitis. In addition, some ideas are proposed to help prevent *E. coli* mastitis and potentially other diseases caused by Gram-negative infections in general.

TLR-4 / *E. coli* mastitis / CD14

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1. INTRODUCTION

Mastitis is the most costly production disease in dairy herds, and when calculated on a per cow within herd basis, mean costs of $40/cow/year are attributed to clinical mastitis [62]. This inflammation of the mammary gland is almost always caused by bacteria (both Gram-positive and Gram-negative) that invade the mammary gland by penetration through the teat canal. The incidence and severity of septic *Escherichia coli* mastitis in dairy cattle is mainly dependent on cow factors [29]. During the periparturient period, the non-specific or innate immunity of the cow is depressed, which makes cows more susceptible to intramammary infection by environmental pathogens like *E. coli*, while cows in mid lactation cure spontaneously from such infections [29]. There is substantial evidence demonstrating that at these periods, the expected influx of polymorphonuclear neutrophil leukocytes (PMN) into the mammary gland is delayed during inflammation after intramammary infection with *E. coli* [60, 61, 159]. In this review, we focus on intramammary infections by *E. coli* during periods when innate defence mechanisms are impaired (i.e., parturition through the first 60 days of lactation).

Currently, prevention of intramammary infection by *E. coli* mastitis is more important than post-infection treatment. Several attempts have been made to prevent cows from contracting *E. coli* mastitis through vaccination [40]. *E. coli* strain J5 is a rough (Rc) mutant strain that consists of a conserved lipid A portion of the cell wall and some common core polysaccharides. Upon vaccination, antibodies against this J5 antigen are generated. The potential mechanism of action of the J5 vaccine has been described by Dosogne et al. (2002) [40]. In some cases, the vaccine has proven to reduce bacterial and somatic cell counts in milk after experimental intramammary *E. coli* challenge [63]. However, although serum and milk antibody titers often increase after vaccination, the expected decrease in incidence and/or severity of *E. coli* mastitis has not consistently been observed [151, 152]. This inconsistency in efficacy could be explained by the fact that the antibody titers in milk might remain below concentrations that are protective against infection. Smith et al. (1999) showed that intramammary immunization enhanced the increase in milk antibody titers compared to subcutaneous immunization [139].

Phagocytosis by PMN is the most effective first line of defence against bacterial infection after penetration of the teat canal [111]. Upon infection of the mammary gland, the influx of PMN needs to rapidly increase in order to clear the mammary gland of invading pathogens. In the 1980's, researchers protected cows from intramammary infection by introducing a polyethylene intramammary device into the mammary gland [24, 111, 112, 118, 132]. The placement of such a device resulted in an increased PMN population in the teat and gland cisterns, thus providing the first line of defence. Although the local presence of PMN in the mammary gland was shown to be beneficial, dairymen need alternative solutions to prevent *E. coli* mastitis, because the somatic cell count is used as a measure of milk quality.

In the gastro-intestinal tract, pathogens trigger a local innate host response by activating the local epithelial cells, and bacterial adherence signaling has been implicated as a key event in this process. However, in the mammary gland, *E. coli* does not seem to colonize in the mammary gland as does *Staphylococcus aureus*. Therefore, the interaction between the *E. coli* and mammary epithelium has received little attention [29]. The discovery of toll-like receptors as evolutionary conservative molecules and their role in innate defence has opened a new area of interest. A number of research groups have reported on the importance of toll-like receptors (TLR) as the first line of defence against invading pathogens through initiation of the innate immune response [1, 73]. The molecular machinery utilized by the mammary epithelial cells to recognize *E. coli* is now under investigation in several laboratories worldwide. The contribution of mammary epithelial cells to the local defence mechanism is also under growing scrutiny [124]. The identification of CD14, TLR-2 and TLR-4 on milk fat globule membranes suggests a direct role for the mammary gland parenchyma in pathogen detection [125]. Biopsies of the

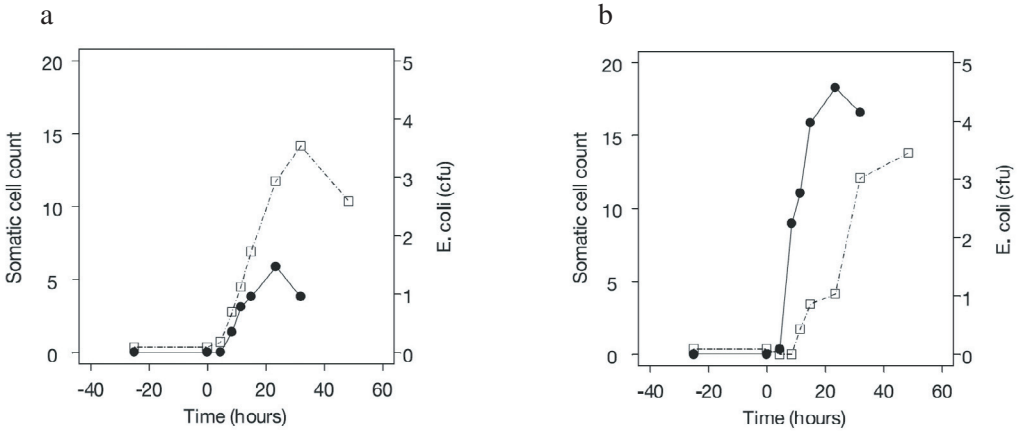

Figure 1. Multiplication rate of *Escherichia coli* (10^6/mL; •) in inoculated mammary glands and pattern of leukocyte influx (10^5/mL; □) into milk in (a) periparturient moderate responders compared with (b) those in severe responders during experimentally induced *E. coli* mastitis in multiparous cows. (Figure taken from the original paper [159], with permission by the Journal of Dairy Research.)

parenchyma of udders with mastitis show increased mRNA abundance of the TLR-2 and TLR-4 genes (not of TLR-9), although the cell types expressing the TLR are yet to be identified [51].

The TLR family of transmembrane proteins plays a key role in recognizing the conserved parts of pathogens and in triggering the innate immune response. Until now, 10 family members have been identified in humans and 11 in mice, of which TLR-4 recognizes the conserved lipopolysaccharide (LPS) pattern of Gram-negative bacteria. TLR-4 may, therefore, play an important role in the innate immune status of cows during periods of risk from intramammary infection by Gram-negative organisms [96]. It has been reported that the actual number of TLR-4 molecules involved in recognition is important for initiation of signaling that leads to activation of the innate immune response [157].

Interestingly, Vangroenweghe et al. (2004) reported that the speed of the inflammatory reaction in primiparous cows is related to the number of *E. coli* bacteria that were inoculated in the mammary gland under experimental conditions: the more bacteria that are invading the mammary gland, the faster they will be eliminated [160]. On the contrary, delays in the inflammatory response in cows with peracute coliform mastitis shortly after calving have been reported [59–61,159] (Fig. 1). Shuster et al. (1996) observed one occasional cow that did not appear to recognize the presence of *E. coli* in the mammary gland and hence allowed the organism to grow to large numbers that ultimately resulted in a fatal case of toxic mastitis. Postpartum cows were much more susceptible to bacterial growth in milk in these studies (100-times higher numbers of *E. coli* were found in postpartum versus midlactation cows), which was consistent with these clinical findings. Based on these facts, one can, therefore, hypothesize that during the periparturient period and during early lactation in multiparous cows but not in primiparous cows, the threshold for LPS sensing is compromised, leading to delayed detection of the invading pathogens. The result of a compromised LPS sensing may be reduced diapedesis of PMN into the mammary gland (that is initiated upon *E. coli* recognition), which results in a more severe form of mastitis. Consequently, the most effective way to prevent mastitis in dairy cattle might be increasing the innate immune response during times of increased risk (during the periparturient period and at early lactation), by increasing the ability of the mammary gland to quickly detect the invading pathogens.

2. IS THE TLR-4 PATHWAY INVOLVED IN *E. COLI* MASTITIS?

2.1. LPS/TLR-4 signaling pathways and their associated cell responses

Sensing the presence of bacteria in the lumen of the mammary gland is an important step in innate immunity. In the bovine mammary gland, it seems that the host response observed after *E. coli* infection is not initiated until bacterial concentrations reach a certain threshold [137]. Because bacterial growth is accompanied by the release of metabolites and toxins, it seems logical that bacteria can be detected by the host through their release of these soluble molecules [124]. Endotoxin or LPS is the major constituent of the Gram-negative bacterial cell wall that is released during growth and death of bacteria. The ability to recognize LPS is an evolutionary mechanism by which the host detects Gram-negative bacterial infections. It is striking to see how a highly conserved molecule, such as LPS, is used by the host to recognize Gram-negative pathogens, and how at the same time this interaction may elicit an adverse immunopathologic effect on the host [30]. TLR-4 has been identified as the pattern recognition receptor (PRR) for LPS that initiates the pathogen-associated molecular pattern (PAMP) for *E. coli*. This was proven by the fact that TLR-4 knock out mice are hyporesponsive and have, therefore, an impaired response to LPS [66, 120, 123]. In general, TLR-4 recognizes LPS and activates a signaling cascade that leads to a variety of host immune responses to the pathogen. With this review, we want to communicate our point of view on the potential involvement of the TLR-4 pathway in the increased susceptibility of cows to *E. coli* mastitis during parturition. In a recent study, Wenz et al. (2006) concluded that *E. coli* strains causing clinical coliform mastitis are of diverse strain types [169]. Although there was no association between strain serotype, genotype, or the presence of specific virulence genes and clinical disease severity, TLR-4 is the common denominator to host cell recognition. However, TLR-4 requires the help of extracellular proteins: LPS-induced signaling is dependent on the formation of the critical receptor complex of TLR-4, CD14 and myeloid differentiation protein 2 (MD-2), which initiates an intracellular signaling cascade that induces an array of responses including the activation of the transcription factor, nuclear factor κB or NF-κB [34].

CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) that exists in a soluble form (sCD14) as well. CD14 is crucial for LPS responses, which has been proven by the fact that CD14-deficient mice are insensitive to LPS [58, 98]. The binding of LPS to mCD14 is greatly enhanced in the presence of the acute phase LPS-binding protein (LBP) [133, 154]. The mCD14-LPS-LBP complex is recognized by TLR-4 (Fig. 2). sCD14 can efficiently present LPS to TLR-4 (or peptidoglycan to TLR-2) on endothelial and epithelial cells that do not express mCD14. The third essential contributor, MD-2 [135] is a protein that is secreted and interacts with the extracellular portion of TLR-4. MD-2 is also found in the Golgi apparatus as a soluble molecule, and has a high affinity to bind both LPS and TLR-4 and is considered to be a possible modulator of the LPS response [135, 163].

The current model states that LPS first binds to CD14, which is optimally achieved in the presence of LBP. The CD14-LPS-LBP complex is presented to the TLR-4-MD-2 complex [154]. LPS recognition by TLR-4 and its associated proteins MD-2 and CD14 leads to the recruitment of the adapter protein myeloid differentiation factor 88 (MyD88) to the intracellular portion of TLR-4 (Fig. 2). It was originally believed that MyD88 recruitment is mediated through direct homotypic binding of respective Toll receptor-interleukin-1 receptor (TIR) domains contained within each protein [92, 100]. However, recent evidence suggests that MyD88 may directly bind to the TIR domain-containing adaptor protein (TIRAP), also known as MyD88 adaptor-like (MAL) protein. MAL directly interacts with the TIR domain of TLR-4 [165]. In the absence of MyD88, TIRAP has been shown to facilitate NF-κB signaling in a MyD88-independent

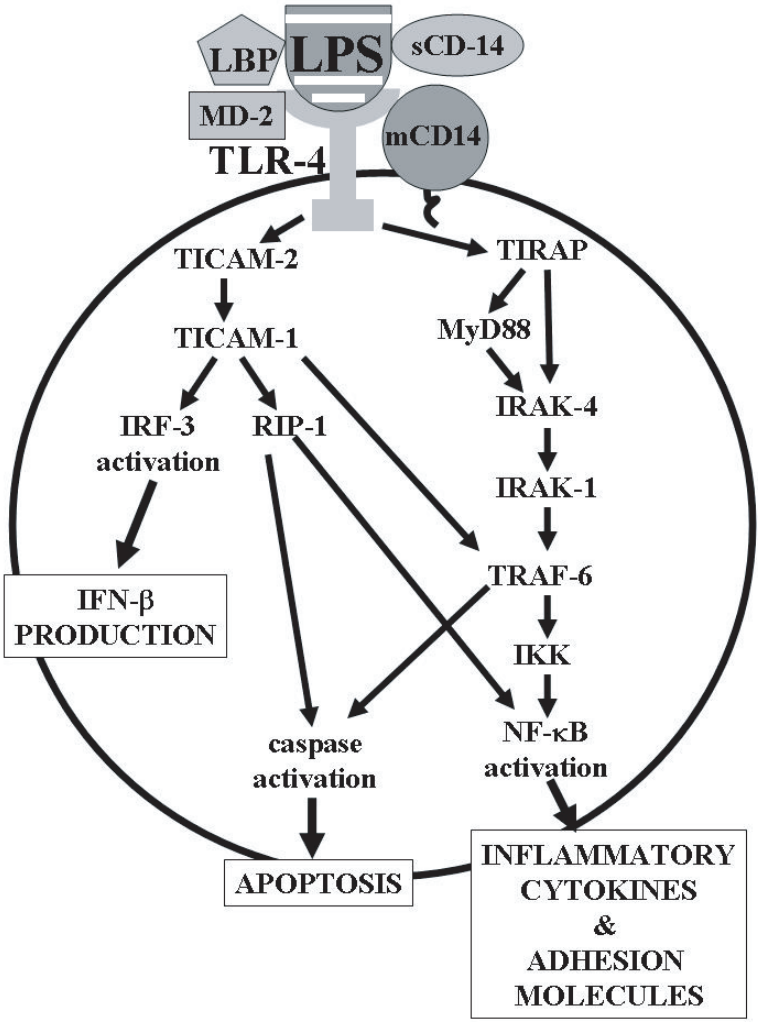

Figure 2. Schematic of TLR-4 signal transduction pathways involved in the induction of cellular inflammatory and apoptotic responses based on human and murine studies. LPS is recognized by a tri-partite recognition complex composed of TLR-4, MD-2, and CD14, the latter of which exerts its role in a cell-type dependent manner as either a glycosylphosphatidylinositol (GPI)-anchored membrane protein (e.g., monocytes) or a soluble protein (e.g., epithelial cells). Recognition of LPS by this multiprotein complex initiates the recruitment of the adapter molecules TIRAP and TICAM-2 to the intracellular domain of TLR-4. TIRAP bridges TLR-4, either directly or through MyD88 to downstream signaling molecules leading to IRAK-4 and IRAK-1 recruitment, kinase activation, and the phosphorylation of IRAK-1. IRAK-1 interaction with TRAF-6 promotes the activation of IKK resulting in NF-κB nuclear translocation and the upregulation of pro-inflammatory cytokines and adhesion molecules. TICAM-2 bridges TLR-4 and TICAM-1 and this signaling pathway has been implicated in promoting NF-κB activation via RIP-1 and/or TRAF-6. Alternatively, TICAM-1 induces the activation of the transcription factor IRF-3 through additional signaling mediators resulting in the upregulation of IFN-β production. In addition, the TIRAP/MyD88/IRAK/TRAF-6 and TICAM-2/TICAM-1/RIP-1 pathways have been implicated in signaling pathways involved in caspase activation and the corresponding induction of apoptosis.

manner [45, 65]. In addition to the TIR domain, human MyD88 contains another highly conserved region referred to as the death domain (DD), which facilitates MyD88's ability to interact with other DD-containing signaling molecules including interleukin 1 (IL-1) receptor-associated kinase (IRAK)-4 [84]. Recruitment to MyD88 facilitates IRAK-4-mediated phosphorylation of IRAK-1. Phosphorylated IRAK-1 subsequently interacts with tumor necrosis factor-α (TNF-α) receptor-associated factor-6 (TRAF-6) and this interaction initiates the activation of a kinase cascade involving IκB kinase (IKK) [31, 75, 83, 146]. Activation of this cascade culminates in the phosphorylation and degradation of the NF-κB inhibitor (IκB), which then enables NF-κB to translocate to the nucleus and promote pro-inflammatory gene expression.

LPS/TLR-4-induced NF-κB activation is not solely mediated through signaling pathways involving TIRAP and MyD88. Following TLR-4 activation, another signaling molecule known as TIR-containing adapter molecule-2 (TICAM-2), also known as TIR domain-containing adaptor-inducing IFN-β-related adaptor molecule (TRAM), is recruited to the intracellular domain of TLR-4 [46, 110, 170]. TICAM-2, in turn, recruits TICAM-1 (also known as TIR domain-containing adaptor-inducing IFN-β (TRIF)), the latter of which induces NF-κB activation via TRAF-6 and/or RIP-1 [95, 129]. In addition to the activation of NF-κB, TICAM-1 and 2 activate the IRF-3 transcription factor, which promotes IFN-β production [134].

In mastitis and other settings where Gram-negative bacteria are the etiological cause of disease, much of the deleterious inflammatory response elicited by LPS is mediated by its ability to activate NF-κB [7, 21, 80, 89]. 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 upregulated by LPS in an NF-κB-dependent manner [19, 39, 52, 108, 136]. Correspondingly, cytokine expression in cows with mastitis has been shown to correlate with NF-κB activation [23]. Besides NF-κB activation, cascades of activation are set in motion by TLR-4 signaling, leading to the activated protein-1, mitogen activated protein kinase (MAPK), and p38. All these signaling pathways result in cellular activation and production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12), oxygen radicals, nitric oxide, tissue factors, as well as anti-inflammatory cytokines like IL-10 and transforming growth factor (TGF). Biological effects induced by LPS are primarily mediated by the cytokines TNF-α and IL-6, which are produced by mononuclear phagocytes and known to be involved in septic shock [22, 78], and by IL-8, which is a chemoattractant for PMN and granulocyte-activating cytokines [105].

TLR-4 signaling cascades not only activate transcription factors, but also active pro-apoptotic signaling that can result in cell death. MyD88, TIRAP, IRAK-1, TICAM-1, TICAM-2, and TRAF-6 have all been implicated in mediating TLR-4-induced apoptosis [12, 13, 67, 72]. There is evidence that the ability of these various TLR-4 signaling molecules to induce apoptosis is mediated through the Fas-associated death domain protein (FADD)-dependent activation of caspase-8 [33, 72]. However, the signaling cascade leading to caspase activation remains to be clearly elucidated.

Furthermore, it should be noted that this model is oversimplified. It has become clear that the recognition and signaling of LPS is not maintained through one single receptor, but through the orchestration of multiple receptors that are concentrated at so-called lipid rafts [155]. Lipid rafts are lateral assemblies of lipids that have a postulated role in the recruitment and concentration of signaling molecules [109]. Several studies hypothesized that additional transmembrane receptors must act in concert with the LPS-CD14 complex to initiate the signaling process leading to LPS-induced cellular activation. CD14-blocking monoclonal antibodies could only partially inhibit LPS-binding, suggesting the existence of alternative receptors [20, 87, 153, 158]. Different signaling molecules must be recruited to

the site of ligation, which is combined with CD14 by LPS. LPS is released into the lipid bilayer where it interacts with a complex of receptors, which include heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), C-X-C chemokine receptor 4 (CXCR4), growth differentiation factor 5 (GDF5) and TLR-4 [153].

For instance, in human monocytes, several LPS-signaling molecules like CD14, hsp70 and hsp90 are constitutively present at lipid rafts [109, 155], while others like TLR-4, CXCR4 and MyD88 are recruited upon LPS stimulation [79, 155, 156]. At these microdomains, receptor molecules are brought into close proximity in order to facilitate protein-protein interactions and subsequent signaling [157]. The cellular responses to different pathogens are controlled by a wide variety of regulatory mechanisms. Depending on the type of pathogen and the cell-type that is being invaded, different supramolecular activation clusters of different receptors are formed, resulting in a signal- and gene-specific activation or repression of transcriptional responses [106, 154].

2.2. Hypothesis: Disturbance of the TLR-4 pathway is involved in the increased susceptibility to *E. coli* mastitis during the periparturient period

When an intramammary *E. coli* infection occurs, bacteria that enter the mammary gland release LPS. As a reaction, a host immune response is initiated where PMN play a pivotal role in clearing the mammary gland by phagocytosing invading bacteria [27]. Interestingly, LPS has been described to enhance the activity and functional life span of PMN by respectively enhancing its oxidative burst activity [48, 126] and inhibiting PMN apoptosis [128].

As described in the previous chapter, LPS is recognized by TLR-4. Therefore it is possible that the TLR-4 signaling pathway is involved in enhancing the PMN activity. It was described that TLR-4 mediates the LPS-induced expanded PMN functional life span [48, 126]. Furthermore, TLR-4 mediates the induction of CD11b/CD18 integrin expression in PMN in response to LPS [86] which is important because CD11b/CD18 adhesion molecules contribute to PMN diapedesis across the bovine blood-milk barrier [140]. The same authors compared CD11b/CD18 expression on PMN from both wild type and TLR-4 deficient mice after LPS stimulation. They observed that in the presence of TLR-4, LPS activated the synthesis and upregulation of CD11b. Therefore, they concluded that TLR-4 plays an important role in PMN adhesion and diapedesis [140, 175].

Recently, Alves-Filho et al. (2006) demonstrated the importance of TLR-4 in PMN migration after Gram-negative infections. They described that upon injection of either LPS or Gram-negative bacteria, an increased PMN migration was demonstrated in TLR-4 wild-type mice, whereas in TLR-4-deficient mice, LPS failed to induce PMN migration [3]. Therefore, we believe that the large influx of activated PMN that enter the mammary gland that resolve the infection, could be a TLR-4-dependent process.

Considering the previous findings, we hypothesize that around parturition, the detection of LPS by TLR-4 may be disturbed. This in turn may result in a delayed or dysfunctional PMN influx into the mammary gland, causing the increased susceptibility for *E. coli* mastitis.

2.3. The link between the TLR-4 pathway and *E. coli* mastitis has been established

The role of other proteins of the TLR-4 pathway in the incidence of *E. coli* mastitis has been studied before. CD14 is a protein that is crucial for LPS responses (see Sect. 2.1). sCD14 occurs in plasma where it helps to convey LPS signaling in cells lacking mCD14 (e.g. endothelial and epithelial cells) (Fig. 3). mCD14 is attached to the cell surface via a glycosyl-phosphatidyinositol tail (GPI) [69], and facilitates LPS recognition and cellular activation despite lacking a transmembrane domain. CD14 is also required for the recognition of other bacterial products including peptidoglycan, lipoteichoic acid (LTA), and lipoarabinomannan [53, 122, 130].

Cells of monocytic lineage and neutrophils express mCD14 and are also a source of shed

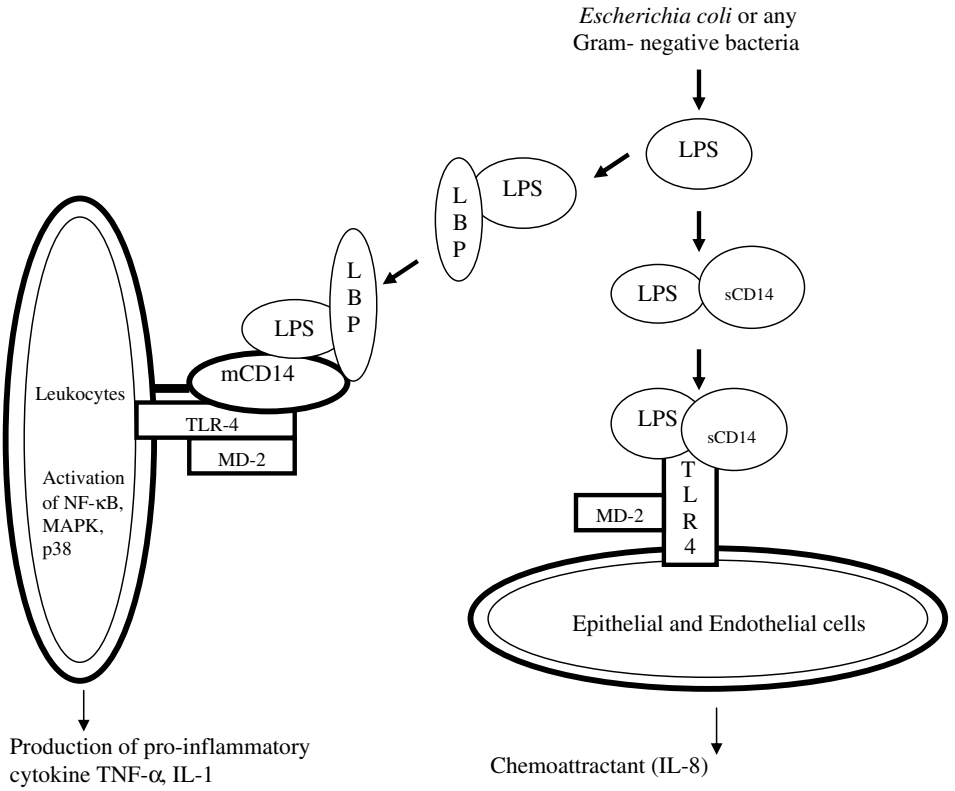

Figure 3. Binding of the LPS-binding protein/LPS complex to mCD14 on monocytes and macrophages causes the release of pro-inflammatory cytokines. Binding of the sCD14/LPS complex to toll receptors on endothelial and epithelial cells causes the release of chemoattracts.

sCD14 [26, 57, 76]. Epithelial and endothelial cells, which are responsive to low levels of LPS only in the presence of exogenous sCD14, were originally believed to be devoid of CD14 expression [8, 49, 121, 149]. More recent evidence suggests that both cell types are capable of expressing CD14 [42,50,77,142]. In light of these recent findings, the need for exogenous sCD14 to facilitate TLR-4 signaling in these cells remains a mystery.

Of relevance to the mammary gland, human and murine mammary epithelial cells have been demonstrated to express CD14 [77, 174], however, whether this protein is expressed in bovine mammary epithelial cells has yet to be reported. Within the healthy mammary gland, resident macrophages and mammary epithelial cells have been postulated to contribute to the basal milk levels of sCD14 as a result of shedding of mCD14 and/or direct exocytosis of CD14 from the Golgi apparatus [77, 162]. During diseases such as mastitis, sCD14 in milk increases following intramammary injection of LPS and various mastitis pathogens [14–16, 81]. The increase in sCD14 parallels the increase of PMN in milk. This suggests that sCD14 was released from the infiltrating PMN. This hypothesis was confirmed by Paape et al. (1996) and Sohn[1] who reported an increase in sCD14 in milk following overnight incubation of PMN in milk,

[1] Sohn E.J., The interrelationship between CD14 and LPS during mastitis: Release of soluble CD14 and cytokines by bovine PMN following activation with LPS, PhD thesis, Univ. MD, College Park, MD, USA, 2005.

or from PMN stimulated with LPS [114]. It was recently shown that the release of sCD14 from PMN causes down-regulation of IL-8 release from PMN. IL-8 is a potent chemoattractant of PMN [5, 53, 115]. Down-regulation of IL-8 by sCD14 suggests a mechanism for controlling excessive migration of PMN into the mammary gland. This is crucial in view of the evidence showing that PMN migration into mammary tissue causes damage to the mammary secretory epithelium resulting in a permanent loss in milk production [105, 113]. In addition, the recombinant (rbos) and plant-derived sCD14 (Prbos CD14) proteins have been described to facilitate the clearance of *E. coli* from the gland when compared to bacterial counts from quarters injected with *E. coli* plus saline [82, 102]. This means that it is possible to increase the innate immune response by locally increasing the sCD14 status in the mammary gland, which could imply that a higher level of sCD14 accelerates the detection of LPS.

Furthermore, it has been shown that intramammary challenge with *E. coli*-derived LPS also induced increased levels of LBP in milk [14], suggesting that the presence of LBP is important for the detection of LPS and for the subsequent response. These results all indicate that the TLR-4 pathway is probably a key element in the detection and subsequent rapid reaction to the invasion of pathogens in the mammary gland.

2.4. What could be a cause for the increased susceptibility of cows to *E. coli* during the periparturient period?

As we previously mentioned, we hypothesize that a high TLR-4 expression level in or on mammary epithelial cells is crucial for detection of invading *E. coli* pathogens and prevention of mastitis in dairy cattle. We hypothesize that during the periparturient period, the detection might be compromised, which can be either caused by downregulated or dysfunctional TLR-4 (e.g. through genetic alterations), or by the masking of LPS.

The expression of TLR is largely restricted to cells of the myeloid lineage, such as dendritic cells and monocytes or macrophages. TLR-4 has also been described to be expressed in endothelial cells, fibroblasts, adipocytes and epithelial cells. Strandberg et al. (2005) described the presence of TLR-4 in bovine mammary epithelial cells [145], an observation that is crucial for our hypothesis. In the period around the partus, the mammary gland has to be provided with an enormous capacity to produce and secrete milk. Because the alveolar cells of the mammary gland undergo drastic biochemical and ultrastructural differentiation for initiation of lactation [161], it is possible that these changes affect the presence or recruitment (discussed in Sect. 3.5) of TLR-4, which is important for the signal that is going to be triggered for recruitment of PMN [157]. Moreover, interfering with the integrity of the lipid raft domains has been reported to inhibit LPS-induced TNF-α production [155]. Therefore, it could be useful to study the presence and/or integrity of the LPS-signaling machinery at these lipid rafts in dairy cattle during the periparturient period and during early lactation.

In addition, the sudden presence of colostrum could play a role as well. Colostrum is known to be one of the most complex body fluids, consisting of over 100 000 molecules. Possibly, lipoproteins present in colostrum mask the presence of LPS. Previous data indicate that lipoproteins neutralize the toxic effects of LPS in vitro as well as in vivo [44, 56].

Current knowledge of LPS/TLR-4 intracellular signaling pathways is based primarily on results from studies in humans and mice (Fig. 2). Those signaling molecules involved in intracellular TLR-4 signaling in cattle have not been well-characterized, and experimental evidence establishing their roles in the bovine innate immune response during mastitis and other Gram-negative bacterial diseases is lacking. Interestingly, there is evidence that cattle are more sensitive to LPS than humans and mice [9, 17, 55] and that there may be differences in the intracellular signaling molecules involved in regulating bovine TLR-4-mediated NF-κB activation. We and others have shown that bovine endothelial cells are directly sensitive to the apoptotic-inducing effects of LPS,

whereas human endothelial cells are resistant unless pre-sensitized with the mRNA or protein synthesis inhibitors, actinomycin-D or cycloheximide, respectively [8–10, 54, 55, 119]. Further, we have demonstrated that relative to its human ortholog, bovine FADD lacks the ability to downregulate TLR-4-induced NF-κB activation [15, 147]. Thus, differences in human and bovine responses to LPS, as well as in the function of signaling molecules that mediate these responses, are known to exist.

TLR-4 and its signaling molecules also play critical roles in host innate immunity. Knockout of, or mutations in, TLR-4 render mice hyporesponsive to LPS and highly susceptible to infection [66, 120, 123]. Human TLR-4 amino acid polymorphisms (like Asp299Gly and Thr399Ile) are associated with diminished pulmonary responsiveness to inhaled LPS and increased susceptibility to Gram-negative bacteremia and septic shock [166]. In addition, mutations in human TLR-4 signaling molecules, including IRAK-4 and other downstream effector proteins that promote NF-κB activation, are similarly associated with an impaired ability to respond to LPS and enhanced susceptibility to infection. The conserved nature of TLR-related molecules in mediating innate immunity in lower species would suggest a critical role for TLR-4 and its signaling molecules across higher order species, including cattle. Thus, identification of genetic variation in bovine TLR-4 and its corresponding signaling molecules may provide insight into susceptibility to mastitis and other bacterial diseases of cattle.

Recently, bovine TLR's and the genes encoding bovine intracellular signaling proteins that facilitate TLR-4-induced NF-κB activation and apoptosis have been cloned, sequenced, and mapped [38, 91, 94, 147, 148]. These studies should facilitate the identification of variations in bovine genes, such as single nucleotide polymorphisms, that are associated with increased disease susceptibility to mastitis and other diseases of cattle. Among those molecules involved in intracellular TLR-4 signaling, amino acid identity between the bovine and human orthologs ranges from 69-92%. Relative to the human orthologs, the predicted functional domains (e.g., death domain regions) within the bovine translated sequences of each gene were found to be highly conserved, suggesting functional similarity of each of the proteins across the two species. Single amino acid changes are reported to ablate the functional capabilities of these signaling molecules [27, 65, 127, 143]. Whether amino acid differences within the bovine TLR-4 signaling molecules affects their function remains unknown. Definitive studies using overexpression, point mutation analysis, and/or dominant-negative strategies will be required to evaluate the role of these proteins in mediating bovine TLR-4-evoked responses. These kinds of tests could help to identify cattle populations at risk.

2.5. Regulation of TLR-4 expression

Several research groups are currently trying to identify the exact mechanism of action of TLR-4. Understanding the mechanism of action of the LPS-recognition and subsequent TLR-4-mediated host response, can teach us to manipulate the response and hence to improve it during periods at risk. Although this is a very complicated field of study, some light has been shed on the signaling mechanisms. Several regulatory mechanisms, like translocation, cleavage and posttranslational modifications (all described below), are candidates for modulating the LPS response. In the following paragraph, we describe some literature and suggestions on the potential mechanism of action on how TLR-4 activity can be enhanced.

Goldammer et al. (2004) reported that TLR-4 mRNA expression was increased in the udder tissue of infected cows, which suggests that TLR-4 is important in the immune response, as a direct or downstream effect [51]. However, microarray experiments in human PMN [88] and human umbilical vein endothelial cells (HUVEC) [173] did not result in the upregulation of any of the genes of the TLR-4 pathway after LPS administration, although the expression of some downstream signaling genes, like IL-8 and NF-κB, was increased. The same results were observed for bovine macrophages [32] and bovine mammary epithelial cells [25, 116]. These results

should be interpreted very carefully, because only one concentration of LPS was used at rather early time points. However, these data do imply that the enhanced TLR-4 activity by LPS is probably not dependent on transcriptional activation, at least not at early time points. This has been confirmed by the group of Franchini et al. (2005) who concluded that the amount of TLR-4 mRNA in bovine macrophages remains relatively constant under a variety of activation conditions [47]. Probably, the recruitment of TLR-4 to the cell membrane happens in another way.

Second, the group of Latz et al. (2002) described that TLR-4 is localized at the plasma membrane and in the Golgi apparatus in human monocytes, and that TLR-4 is able to recycle between those two places [79]. Moreover, MD-2 has been described to play a crucial role in this process [101, 107]. But according to Latz et al. (2002), the internalization and trafficking of TLR-4 to the Golgi apparatus is not necessary to induce LPS signaling, because depletion of the Golgi apparatus did not prevent induction of LPS signaling. Moreover, LPS signaling was initiated upon binding of TLR-4 to an immobilized antibody (when neither internalization nor trafficking to the Golgi complex is possible). In addition, upon LPS exposure, this group has reported that MyD88 – one of the TIR-domain-containing adaptor molecules that binds TLR-4 (see Sect. 2.1), translocates to the surface of the cell [79]. They concluded that in human monocytes the primary site of LPS signaling is located on the plasma membrane, and is further supported by the data of Moller et al. (2005) who described that surface TLR-4 expression on human monocytes is enhanced after LPS stimulation [97]. In contrast, Hornef et al. (2003) described that in epithelial cells of the murine intestinal crypt, the recognition of LPS by TLR-4 occurs intracellularly in the Golgi apparatus [64]. They also describe that upon depletion of the Golgi apparatus, the transfer of LPS to the Golgi apparatus is abolished, but does not impair the subsequent recognition and signaling process. Recently, TLR-4 has been described to be the LPS signal, but not the LPS uptake receptor [41], confirming the predominantly signaling function of TLR-4. Taken together, both groups describe the localization of TLR-4 at the Golgi apparatus, but neither of them can explain the functionality of this phenomenon. Both groups agree on the fact that the blocked transfer of LPS to the Golgi does not impair initiation of LPS signaling. Likewise, TLR-4 has been described to be present on the membrane of monocytes, but not on the membrane of epithelial cells of the murine intestinal crypt.

It is possible that the Golgi pool of TLR-4 serves as a steady state pool of TLR-4 and that by means of TLR-4 trafficking to and from the plasma membrane, the proper surface expression of TLR-4 can be regulated. This regulatory mechanism is only possible if the cells express TLR-4 on their membrane [79] at the so-called lipid rafts.

In conclusion, the regulation of the TLR-4 signaling pathway probably occurs only minimally at the transcriptional level and is mainly controlled at the protein level, making it a very fast and dynamic process. The clue will be to understand how the TLR-4 trafficking to and from the cell membrane is regulated in order to manipulate it and hence lower the threshold for the detection of LPS as much as possible.

2.6. Tools to further study the involvement of the TLR-4 signaling pathway in the incidence of *E. coli* mastitis during the periparturient period

One of the difficulties in studying the mechanism of action of the TLR-4 regulatory mechanism, is the fact that it is technically challenging to detect TLR-4 protein expression, and consequently to monitor changes in TLR-4 protein expression or TLR-4 cellular translocations as well. As a downstream read-out of TLR-4 activation, production of cytokines or increased NF-κB activity is often measured.

To study TLR-4 directly, in vitro experiments are the obvious way. Several useful techniques have been developed. Reducing the endogenous TLR-4 or any other participant of the pathway, using the technique of RNA interference, could reveal the importance of the presence of TLR-4 on bovine mammary epithelial cells. In contrast, overexpressing the

bovine TLR-4 in bovine mammary epithelial cells, could determine how the receptor functions. Tagged TLR are often cloned and used to study the pathway because antibodies are not well established. TLR-4 has been reported to give poor Western blot results: Nishiya et al. (2006) described that yellow fluorescent protein (YFP)-tagged TLR-4 appeared as a smeared band in the high molecular range on Western blot, which is probably caused by TLR-4 oligomerization and aggregation [103]. Furthermore, it is recognized that binding of TLR-4 to a specific antibody either activates the signaling pathway [79] or blocks it [97]. Therefore, it would be advised to use other detection methods besides Western blot or immmunocytochemistry, such as confocal microscopy and monitor a tagged receptor instead of using antibodies. For instance, tagging TLR-4 with a fluorescent probe (cf. the experiment of the group of Latz et al. (2002) [79] or Husebye et al. (2006) [68]) makes it possible to follow the intracellular trafficking under basal conditions or upon stimulation with LPS or other compounds. Therefore, bovine TLR-4 has to be cloned in frame with a reporter gene like green fluorescent protein (GFP) in such a way that a fusion protein is translated encoding for the GFP tagged-receptor. It is, however, crucial to check the functionality of this receptor (is it properly folded and distributed in the cell?) because it is possible that the tag can interfere with the receptor's functionality [103].

In addition, we should keep in mind that in vitro systems are not always predictive for what will happen in vivo. In an in vitro setup, the physiological regulatory systems of the cow are absent, which on the one hand reduces the amount of variables, but on the other hand, reduces the resemblance with the in vivo situation. Therefore, the in vitro system is useful to prove the mechanism of action of compounds that could protect dairy cattle against *E. coli* mastitis by activating the TLR-4 pathway.

3. THE USE OF TLR-4 AGONISTS TO PREVENT *E. COLI* MASTITIS

3.1. TLR-4 agonists

It is our intention to increase the dairy cow's first line of defence against invading *E. coli* pathogens without increasing the milk somatic cell count (SCC), i.e. by means of vaccination. Supposing that the TLR-4 pathway is dysfunctional or depressed around parturition, TLR-4 agonists could be used to restore or activate the TLR-4 signal transduction pathway and enhance the subsequent PMN recruitment. Several research groups have made some attempts to sensitize the detection of LPS. Preventive administration of LPS has been done before as described by Singh et al. (2000) who used LPS as an intrauterine immunomodulator to cure bacterial endometritis in cattle [138]. Intrauterine administration of LPS increased the local amount of leukocytes, of which 80% were PMN. However, the use of endotoxins in vaccines is limited due to the undesirable side effects, like fever and local inflammation [18]. Therefore, people are looking for some good alternatives, such as compounds that have the immunogenicity of LPS, but lack its toxicity.

Nowadays, new types of compounds are being developed, namely TLR-4 agonists that mimick the LPS structure and are able to activate the innate immune response. These compounds possess the immunogenic properties of LPS but lack its toxicity. Monophosphoryl-A (MLA), a non-toxic variant of the lipid A portion of LPS that lacks the (R)-3-hydroxytetradecanoyl group and 1-phosphate, has been developed [90]. LPS and MLA induce similar cytokine profiles, but MLA is at least 100-fold less toxic [36]. Recently, aminoalkyl glucosaminide phosphates (AGP) have been designed and synthesized. They belong to a family of lipid A mimetics, and have been shown to induce innate immune responses in a TLR-4 dependent manner: intranasal challenge with influenza virus resulted in a 70% survival rate in wild type mice that were pretreated with AGP, but not in the C3H/HeJ TLR-4 deficient mice [36]. Accordingly, microarray results showed that the TLR-4 agonists clearly enhanced gene expression of cytokines like IL-6 and TNF-α, and transcription of NF-κB in human macrophages that were stimulated for 6 h with AGP [144]. In numerous preclinical and clinical studies, MLA and AGP have been shown to be potent yet non-toxic vaccine adjuvants [117]. They

are being used as stand-alone therapeutic immunomodulators as well. Both agonists stimulate production of soluble mediators, including cytokines and chemokines, which recruit and activate a broad array of immune effector cells, and enhance cellular interactions, cooperation and overall immune function [6].

Therefore, it would be of interest to investigate the effect of intramammary administration of TLR-4 agonists to the dairy cow before or during the periparturient period at risk in order to provide protection against infection by *E. coli*.

3.2. Administration of TLR-4 agonists

The main question to be addressed is how should such a TLR-4 agonist be administered: locally or systemically? Recently, Andonegui et al. (2003) did a very interesting study to address the question: how important is the expression of TLR-4 on circulating PMN and on lung endothelial cells for PMN sequestration in the lungs upon infection? They produced TLR-4 knockout mice transplanted with leukocytes expressing TLR-4 (endothelial cells are TLR-4$^-$ and PMN are TLR-4$^+$) and TLR-4 wild type mice transplanted with leukocytes knocked out for TLR-4 (endothelial cells are TLR-4$^+$ and PMN are TLR-4$^-$) [4]. They suggested that TLR-4 expression was not required on PMN for their sequestration into the lungs. On the contrary, they claimed that the activation of the lung endothelium is indeed dependent on the endothelial TLR-4 expression. Applied to our mastitis model, these results suggest that the expression of TLR-4 is more important on the epithelial cells of the mammary gland than on the PMN that have to be recruited, implying that local administration would be the route of choice. However, local administration (i.e. intramammary) is probably not an option because it is our goal to maintain a low milk SCC. Intramammary administration would probably induce a PMN influx in the mammary gland, thus increasing the milk SCC.

Systemic administration of the TLR-4 agonist should provide the cow with an overall increased state of innate immunity. But what will happen with the PMN if they have an increased TLR-4 expression? It has been described that upon systemic activation of TLR-4 (LPS administration), leukocyte rolling mechanisms were down-regulated [74], and no transmigration of PMN to the peripheral tissues was observed. In that study, LPS, and not a non-toxic TLR-4-agonist, was used to activate TLR-4.

We hypothesize that systemic administration of TLR-4 agonists will certainly be beneficial for the global innate immune status of the animal and will protect the dairy cow not only against severe *E. coli* mastitis, but also against other infection diseases (like endometritis) or allergies.

3.3. Possible drawbacks of the therapy

Although TLR-4 agonists are already being used as immunomodulators, we should take into account that the preventive administration of TLR-4 agonists to the dairy cow has some putative drawbacks.

Pretreatment of cells with LPS results in LPS tolerance, meaning that cells will react less to LPS after pre-exposure. However, the underlying molecular mechanisms that induce LPS tolerance remain to be resolved. MyD88s may play an important role in this process. MyD88s is a splice variant of MyD88 (one of the TIR-domain-containing adaptor molecules that binds TLR-4) and has an antagonistic activity on the TLR-4 signaling pathway. MyD88s has been reported to be expressed under chronic conditions of inflammation or after prolonged exposure [71]. In addition, besides upregulation of MyD88s after LPS pre-exposure, it has been reported in mouse macrophages that TLR-4 surface expression is down regulated [104], which is a disadvantage because a certain threshold needs to be reached in order to induce signaling (described in the introduction).

Other cellular mechanisms have been described that can counteract the therapy by providing a negative feedback regulation after overexpression of TLR-4. An alternatively spliced form of TLR-4 has been identified in mice and humans and was named mTLR-4

(mouse) [70] or sTLR-4 (human) [94]. These other forms of TLR-4 are soluble and lack the putative intracellular and transmembrane domains. Soluble TLR-4 neutralizes LPS-induced inflammation in TLR-4-expressing cells and inhibits an excessive LPS response [70]. This implies that TLR-4 may be functionally regulated by alternative splicing in various species. However, it should be noted that it is not LPS that will be exposed to the body, but a mimicking chemical compound, which means that a completely different reaction could take place. However, this remains to be investigated.

Based on the results of Alves-Filho et al. (2006) [3], a comment should be made regarding the inflammatory events during lethal polymicrobial sepsis (infection by two or more different types of bacteria) [2]. They induced lethal polymicrobial sepsis by caecal ligation and puncture (CLP) in wild type and TLR-4 deficient mice and describe that in wild type mice, there is a clear PMN migration failure to the infectious focus compared to the TLR-4 deficient mice. Because this was the opposite reaction of what was observed after Gram-negative infections, this could be due to the presence of other (Gram-positive) microbial components. This means that TLR-4 agonists are beneficial in cases of Gram-negative infections, but can be lethal in cases of polymicrobial infections. These results emphasize that the line between enhancing innate immune response and preventing septic shock is a very fine line. An overdose of endotoxin can sometimes induce severe shock [18]. Therefore, it will be challenging to draw the line between enhancing the innate immune response by TLR-4 agonists and inducing septic shock. During early infection events, TLR-4 should be upregulated and ready to detect the invading pathogens. The therapy with TLR-4 agonists that we propose is meant to be administered preventively, before infection takes place. During later inflammatory events, TLR-4 upregulation can induce severe septic shock, which is demonstrated by the fact that TLR-4-directed LPS antagonists are used as a treatment for diseases that are caused by endotoxins (like septic shock) [99, 150].

3.4. Alternative ways to activate the TLR-4 signaling pathway

3.4.1. Induction of TLR-4 expression

Besides using TLR-4 agonists, there are other ways to stimulate TLR-4 expression. One of the possibilities is to use radiodetoxified (RD) LPS (ionizing radiation of LPS), which decreases LPS toxicity but preserves the beneficial effects of LPS (reviewed in [18]). RD-LPS increases the natural innate immunity in vaccinated animals [18]. First of all, LPS has been shown to induce inducible nitric oxide synthase (iNOS) production, which contributes to the LPS toxicity. Pretreatment of macrophages with RD-LPS induces tolerance of the cells towards NO induction by toxic LPS. Second, like LPS, RD-LPS induces CD11b and CD18 integrin expression on PMN [37]. Furthermore, RD-LPS reduces lethality in mice after induction of bacterial peritonitis [37]. And third, RD-LPS increases the production of white blood cells in healthy animals [37], which might be an extra benefit because immediately after infection, a systemic leukopenia is established [93].

Another possibility is to induce MD-2 secretion. It has been reported that MD-2 binds to the extracellular domain of TLR-4 and causes their surface expression levels to increase [43]. MD-2 is crucial for the translocation of TLR-4 from the Golgi apparatus to the cell surface [101, 107]. In this way, it can manipulate the expression of TLR-4 on the cell surface, making this a positive feedback loop to regulate TLR-4 expression levels [43]. MD-2 is found as well in the Golgi apparatus as a soluble molecule and has a high affinity to bind both LPS and TLR-4 and is therefore a possible modulator of the LPS response [135, 163]. This means that MD-2 is not only crucial for LPS binding to TLR-4 but also for its expression levels. MD-2 could therefore be a key candidate to modulate LPS responses through the TLR-4 pathway by interfering with the machinery that regulates the membrane trafficking of TLR-4. Recently, it has been suggested that by targeting MD-2, endotoxin responses can be manipulated [164].

Various other potential drug targets to enhance TLR-4 signaling are the negative

regulators that can inhibit or terminate TLR-4-mediated immune responses like MyD88 (reviewed by Liew et al. (2005) [85]). Likewise, TLR-4 expression can also be regulated by ubiquitination because TLR-4 degradation is promoted by Triad3A, an E3 ubiquitin-protein ligase, that ubiquitinates it [35]. Interestingly, besides targeting proteins for proteolytic degradation, ubiquitination also regulates membrane trafficking of receptors, making it a very interesting regulatory factor for TLR-4. Very recently, the group of Husebye et al. (2006) [68] reported that the LPS receptor complex can terminate LPS signaling through the endosomal trafficking of the complex and subsequent degradation. Besides terminating the first innate immune responses, the LPS receptor complex is also involved in LPS-associated antigen presentation to $CD4^+$ T cells, which means that TLR-4 is involved in the control of the adaptive immune system as well, which makes it an even more interesting drug target for induction of the immune responses [68].

3.4.2. Induction of CD14 expression

In the past several years, recombinant nucleocapsid proteins have been expressed in *E. coli,* in a baculo- and vaccinia-virus expression system in silkworms and in mammalian cells [131]. Recombinant nucelocapsid proteins expressed in insect cells, bacteria or as chimeric hepatitis B virus (HBV) core particles, can evoke protective immunity in an animal model [171]. The gene responsible for production of bovine CD14 has been cloned [168], and a recombinant virus containing the N-terminal 1 – 358 amino acids of bovine CD14 (rbosCD14) was generated by co-transfection of insect sf-9 cells with the clone and purified.

To further characterize the chemical and biological properties of rbosCD14, a panel of ten murine monoclonal antibodies (mAb) reactive with recombinant bovine CD14 (rbos) were produced [141]. A sandwich enzyme-linked immunosorbent assay (ELISA), using murine mAb and rabbit polyclonal antibodies reactive with rbosCD14 was developed. All the mAbs were reactive by ELISA with baculovirus-derived rbosCD14 and they recognized rbosCD14 (40 kDa) by Western blot analysis. The mAb also identified by Western blot sCD14 (53 and 58 kDa) in milk and blood and mCD14 (47 kDa) in a lysate of macrophages obtained from involuted bovine mammary gland secretions. Analysis by ELISA of whey samples after intramammary injection of LPS (10 μg) revealed increased sCD14 levels between 8 and 48 h after injection. Flow cytometric analysis showed that the mAb bound to macrophages isolated from involuted mammary gland secretions and mouse macrophages but not to swine or horse monocytes. Addition of anti-rbosCD14 mAb to monocytes stimulated with LPS reduced in vitro production of TNF-α.

Studies have also shown that proteins expressed in plants are effective tools for production of antigens. Bacterial toxins and various viral proteins have been successfully expressed in plants. These proteins were able to elicit specific humoral and mucosal immune responses when administered intraperitoneally and protected animals against the corresponding bacterial or viral infections [172]. Recently, rbosCD14 was directly cloned into a plant virus, and used to infect tobacco plants [102]. The plant-derived sCD14 (PrbosCD14) was purified from plant extracts.

Both the rbosCD14 and PrbosCD14 showed good biological activity in dairy cows. When both recombinant proteins where individually combined with LPS and injected into udders of lactating dairy cows, the resulting increase in milk somatic cells for mammary quarters receiving the recombinant proteins and LPS were greater compared to somatic cell counts for quarters injected with just LPS [82, 102, 167]. Further, quarters injected with the recombinant proteins and challenged with *E. coli* had reduced clinical symptoms, such as mammary swelling and abnormal milk, compared to *E. coli*-challenged quarters injected with saline [82, 102]. Both recombinant proteins also facilitated the clearance of *E. coli* from the gland when compared to bacterial counts from quarters injected with *E. coli* plus saline. These results suggest that rbos and Prbos can be potent prophylactic

tools for reducing severity of infection by Gram-negative organisms.

4. CONCLUSION

E. coli infections of the mammary gland frequently occur in dairy cattle at periods when the innate defence mechanisms are impaired: during the periparturient period and during early lactation. Currently, prevention of mastitis by *E. coli* is more important than post-infection treatment. Several unsuccessful attempts have been made to prevent cows from this Gram-negative infection. Without doubt, the prevention of *E. coli* mastitis is a complicated matter, especially when one takes into account the maintenance of a low milk SCC.

We hypothesize that in dairy cattle during the periparturient period and during early lactation, the detection of invading pathogens might be impaired and, therefore, the diapedesis of PMN into the mammary gland during infection is altered, which results in a more severe form of mastitis. Since the TLR-4 receptor is crucial for detecting the presence of *E. coli*, a dysfunctional TLR-4 pathway might be the reason for the impaired PMN influx. However, other mechanisms of action should not be forgotten.

Until now, most research has been focussed on the efferent (or effector) arm of the innate immune system in search of the dysfunctional trigger that should normally eradicate the *E. coli* infection. In this field, PMN functionality has been one of the main focuses. Recently, the effect of glucocorticoids on PMN gene expression was studied describing a possible influence of glucocorticoids on PMN functionality and hence a possible explanation for the increased mastitis incidence during the periparturient period [28]. Although most results have been booked in this research area, we have focused on the possible contribution of the afferent (or sensing) arm in the pathogenesis of *E. coli* mastitis. Our hypothesis states that the TLR-4 pathway might be impaired or dysfunctional during the periparturient period and that understanding the regulatory mechanisms by which the TLR-4 pathway transduces the LPS signal after infection, is crucial in order to manipulate the signal transduction. However, to prove our hypothesis, a lot of research needs to be done: (i) investigating the exact mechanism of action of LPS detection in the mammary gland i.e. which cell type, which receptor complexes, etc. (is it TLR-4 mediated, is the TLR-4 pathway impaired during the periparturient period?), (ii) understanding the regulatory mechanisms by which this mechanism of action is controlled (posttranslational modifications? Certain protein-protein interactions?), and (iii) investigate whether this mechanism can be manipulated in order to restore the immunosuppressed conditions of the cow during the periparturient period (can we use TLR-4 agonists to restore mammary gland reactivity?).

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