Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with *Escherichia coli*

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ABSTRACT

The objective of the current study was to investigate the toll-like receptors (TLR), including the soluble forms sTLR2 and sTLR4, involved in innate immune responses of dairy cows to experimentally induced *Escherichia coli* mastitis. Six clinically healthy Holstein dairy cows received an intramammary inoculation of *E. coli* O111:K58 between 63 and 83 d postpartum. Concentrations of sTLR2 and sTLR4, the proinflammatory cytokines IL-6 and tumor necrosis factor- α (TNF- α), and acute phase proteins serum amyloid A (SAA) and haptoglobin (Hp) in blood were measured by ELISA. Furthermore, 10 mL of milk was collected from challenged quarters immediately before inoculation and at 6, 12, 24, 48, and 72 h after inoculation, and mRNA expression of selected genes, including TLR2, TLR4, IL-1 β , IL-6, TNF- α , and IL-8, was quantified by real-time PCR. *Escherichia coli* intramammary infection elicited a decrease in the circulating levels of leukocytes. Rectal temperature was elevated at 6 h postinoculation (PI). Similarly, the serum concentrations of TNF- α , IL-6, and SAA increased at 6 h PI. However, serum concentrations of sTLR2, sTLR4, and Hp did not differ after challenge. The mRNA expression of TLR2, IL-1 β , and IL-8 in milk somatic cells increased at 12 h PI, whereas a decreased IL-6 mRNA expression was detected from 6 to 48 h PI. In conclusion, we found that TLR2 mRNA expression increased in milk somatic cells collected from infected quarters of cows challenged with *E. coli*, whereas the concentrations of sTLR2 and sTLR4 remained unchanged after challenge. Thus, sTLR2 and sTLR4 may protect the host by sequestering pathogen-associated molecular patterns during *E. coli* mastitis.

Key words: bovine mastitis, cytokine, *Escherichia coli*, toll-like receptor

INTRODUCTION

Bovine mastitis, which is an inflammation of the mammary gland that often develops following intramammary bacterial infection, is one of the most prevalent and economically costly diseases to the dairy industry (Seegers et al., 2003). Gram-negative bacteria are responsible for approximately one-third of all clinical cases of bovine mastitis, and almost 25% of these cases result in culling or death of the animal (Eberhart, 1984). Among the gram-negative bacteria, *Escherichia coli* remain the most prevalent cause of clinical mastitis in cows (Wilson et al., 1997).

The innate immune system provides initial protection against *E. coli* infection by detecting the presence of the invading pathogens and mounting an inflammatory response (Akira et al., 2006). Recognition of pathogens is mediated by cellular pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMP; Aderem and Ulevitch, 2000). Toll-like receptors (TLR) comprise a family of pattern recognition receptors that are capable of recognizing distinct PAMP (Hallman et al., 2001). For example, TLR2 recognizes peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* and other gram-positive bacteria, whereas TLR4 recognizes LPS from gram-negative bacteria, including *E. coli* (Hoffmann et al., 1999). In cattle, sequences for 10 TLR have been described, and each TLR is capable of recognizing a distinct PAMP (Werling et al., 2006). Expression of both TLR2 and TLR4 mRNA increases in mammary glands of cows with mastitis caused by either *Staph. aureus* or *E. coli* (Goldammer et al., 2004). Likewise, an increase in TLR2 mRNA expression was found in infected mammary glands of cows with experimentally induced *E. coli* mastitis (Petzl et al., 2008). Although most TLR are transmembrane receptors, soluble forms of some TLR (sTLR) have recently been identified in various body fluids and cellular secretions in human (Srinivasan et al., 2008; Dulay et al., 2009; Zunt et al., 2009), although little is known of their role in domestic animals.

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A key component of the host innate immune response to infection is the upregulation of cytokine production (Dinarello, 1996b; Koj, 1996; Suffredini et al., 1999). Cytokines such as tumor necrosis factor- α (TNF- α), IL-1, and IL-6 mediate the inflammatory response at both the local and systemic levels and are regarded as proinflammatory cytokines (Dinarello, 1996a; Thijs et al., 1996). Locally, these cytokines induce expression of vascular endothelial adhesion molecule, thereby promoting neutrophil transendothelial migration to the site of infection. Systemically, TNF- α and IL-1 β are potent inducers of fever and the acute phase response, which is characterized by fever, leukocyte mobilization, and increased production of inflammatory cytokines and acute phase proteins (APP) such as serum amyloid A (SAA), haptoglobin (Hp), and LPS-binding protein (Uthaisangsook et al., 2002). Increases in serum or plasma concentrations of TNF- α , IL-1 β , or IL-6 have been reported during mastitis in cows (Bannerman et al., 2004). Moreover, previous studies in cows (Lee et al., 2006) have detected significant increases in IL-1 β , IL-6, IL-8, and TNF- α at both the mRNA and the protein levels in either milk or mammary tissues collected from the infected mammary glands following intramammary inoculation with *E. coli*.

Therefore, the aim of this study was to investigate mRNA expression of TLR2 and TLR4 in milk somatic cells, and their soluble forms sTLR2 and sTLR4 in blood, during innate immune responses of dairy cows to experimentally induced *E. coli* mastitis.

MATERIALS AND METHODS

Cows

Six clinically healthy Holstein dairy cows at a commercial dairy herd in Beijing were selected for the study. Cows (3 to 4 yr old and 63 to 83 d postpartum) were free of pathogens through 3 consecutive bacteriological examinations and each had quarter milk SCC <200,000 cells/mL 10 d before the start of the trial. Cows were kept in individual box stalls and fed a mixed ration formulated for the dairy herd. They were milked twice daily at 0700 and 1800 h. Daily milk production of each cow (kg/d) was measured. The use and care of all animals in this study was approved by the China Laboratory Animal Care and Use Committee.

E. coli Strain

The *E. coli* strain O111:K58 (CVCC1450; China Institute of Veterinary Drug Centre, Beijing, China) used for the induction of mastitis (Zhen et al., 2008) was grown in Luria-Bertani broth containing 1% tryptone,

0.5% yeast extract, and 1% NaCl, at pH 7.0. Tryptone and yeast extract were from Oxoid (Basingstoke, UK). After overnight incubation at 37°C with shaking, bacteria were diluted 1:100 in fresh Luria-Bertani broth. Following incubation, the bacterial cells were harvested by centrifugation at $3,000 \times g$ for 10 min at 4°C, washed in sterile physiological saline, and resuspended in saline. Inoculum dose was generated as described in a previous study (Vangroenweghe et al., 2004). A solution of *E. coli* containing approximately 1×10^6 cfu/mL was prepared. Concentrations of *E. coli* were determined by densitometry followed by counts of colony-forming units after agar plating of bacterial serial dilutions.

Experimental Design and Intramammary Challenge

The teat of the left rear quarter of each cow was carefully washed with soap and disinfected with ethanol 30 min after the morning milking. A blunt cannula was then introduced through the teat canal, and 2 mL of *E. coli* inoculum was infused.

Blood and Milk Sampling

Blood samples were taken at the same time points as the milk samples. Blood and milk samples were collected at 0 (just before inoculation), 2, 4, 6, 12, 24, 48, and 72 h after inoculation with *E. coli*. Blood was collected via jugular venipuncture into glass tubes that contained either no additive or EDTA as an anticoagulant, which allowed us to obtain serum and plasma, respectively. Blood samples were centrifuged ($2,000 \times g$ for 30 min at 0°C) and the plasma and serum were stored at -80°C until subsequent analysis. Serum was separated for later quantification of cytokines, APP, and sTLR. Blood sampled with EDTA was used for leukocyte analysis. Ten milliliters of milk from each quarter was collected aseptically for bacteriology and SCC determinations. Total milk was then collected from each quarter immediately before inoculation and at 6, 12, 24, 48, and 72 h after inoculation. All samples were kept on melting ice (1°C) during transport and at the laboratory until analyses were performed or aliquots were prepared for freezing. Milk samples collected were immediately used for RNA extraction and reverse transcription.

Analytical Methods for Indicators of Inflammation

The number of *E. coli* (cfu/mL) after experimental inoculation was determined by appropriate 10-fold dilutions of each milk sample in sterile saline. Ten microliters of each dilution was plated on eosin methylene blue agar (Oxoid), and all dilutions were performed in duplicate. Colonies were counted after 24 h of in-

cubation at 37°C. The colony count was converted to colony-forming units per milliliter based on the factor of dilution and expressed as log₁₀ cfu/mL for statistical analysis. Milk SCC was determined using a cell counter (C-Reader System model ADAM, Digital Bio Technology Inc., Seoul, Korea).

Total white blood cell counts were determined using an automated hematology analyzer (Celltac α, Nihon Kohden, Tokyo, Japan) calibrated for bovine blood. Differential leukocyte counts were estimated by staining of blood smears with May-Grünwald-Giemsa stain and by sequentially counting 200 cells under a microscope using 50-fold magnification.

Serum concentrations of IL-6, TNF-α, and cortisol were determined in duplicate using commercially available bovine ELISA kits (RapidBio Lab, West Hills, CA) with sensitivities of 30 pg/mL, 5 pg/mL, and 5 ng/mL, respectively. Serum concentrations of SAA and Hp were determined in duplicate using commercially available bovine ELISA kits (ESB Laboratory, Sacramento, CA) with sensitivities of 0.5 µg/mL and 0.12 µg/mL, respectively. Soluble TLR2 and sTLR4 were quantified by using biotin-conjugated polyclonal antibody preparations specific for the quantitative determination of bovine TLR2 and TLR4 concentrations in serum and plasma using commercially available ELISA kits (ESB Laboratory) with sensitivities of 0.39 ng/mL and 0.39 ng/mL, respectively.

Milk Somatic Cell Isolation and Viability Test

Milk somatic cell isolation and viability tests were carried out as described previously (Lee et al., 2006) with modifications. Aseptically collected milk samples were diluted 1:1 with cold (4°C) sterile PBS (pH 7.4) and centrifuged (1,000 × *g* for 20 min at 4°C). The fat layer and supernatant were removed and the cell pellet was resuspended and centrifuged twice in PBS (400 × *g* and 300 × *g* for 10 min at 4°C). The final pellet was resuspended in cold PBS (1 to 50 mL, depending on the original total cell concentration, to obtain an optimal cell concentration for differentiation). The cell suspension was used for determining the cell count and viability in a Neubauer counting chamber with direct light microscopy using Trypan blue staining (1:1 dilution). Viable cells appeared shining white under the microscope, whereas dead cells appeared blue.

RNA Extraction and Reverse Transcription

Total RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The optical density at 260:280 ratio of samples was measured with a NanoDrop ND-

1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) to determine the quality of extracted RNA, and the integrity was analyzed by agarose gel electrophoresis through staining with ethidium bromide and visualization under UV light. The RNA was then reversed transcribed into cDNA using the ImProm-II Reverse Transcription Kit (Promega, Madison, WI) according to the manufacturer's instructions. The reverse transcription (RT) reaction was started by adding 2 µg of total RNA and 0.5 µg of oligo (dT₁₅) to 12.5 µL of sterile, distilled water and heating at 72°C for 5 min. After cooling on ice, 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 5× first-strand buffer, and 200 U of Superscript II RNase H–Reverse Transcriptase were added. The mixture was stabilized at 25°C for 10 min and subsequently incubated at 42°C for 60 min for the RT reaction. Thereafter, the temperature was increased to 70°C for 15 min to inactivate the reverse transcriptase. Synthesized cDNA was kept at –20°C until further analysis.

Lightcycler Real-Time PCR

Briefly, the Lightcycler real-time PCR was carried out as described previously (Griesbeck-Zilch et al., 2008) with modifications. Primers for specific bovine genes, as listed in Table 1, were synthesized (Sangon, Shanghai, China) to have an equal annealing temperature of 60°C. The reaction conditions for each individual gene were optimized using a QuantiTect SYBR Green PCR kit (Qiagen, Beijing, China) in the ABI 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA) and applied to the following protocol. The cDNA was analyzed in a 20-µL PCR mixture containing a final concentration of 0.5 µM primer, 2 µL of cDNA, 10 µL of 2× SYBR Premix DimerEraser, and 0.4 µL of ROX (passive reference dye). The PCR mixture was added to a cold PCR capillary, centrifuged, and placed into the LightCycler system. The LightCycler was programmed in 4 steps: (1) denaturation at 95°C for 30 s; (2) amplification for 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; (3) melting curve by 95°C for 15 s, 60°C for 1 min, and 95°C for 30 s; (4) cooling at 40°C. In each reaction, the cycle number at which the fluorescence rose appreciably above the background fluorescence was determined to be the crossing point (cycle threshold or C_T value).

Data Analysis of Real-Time PCR

To evaluate the relative quantification of mRNA expression, the C_T values of the target genes were normalized against the arithmetic mean (AM) of 3 reference

Table 1. Sequences and accession numbers of oligonucleotide primers used for real-time PCR and the length of the PCR products¹

Gene ²	Forward/reverse	Oligonucleotide sequences (5' to 3') of primers	GenBank accession no.	Product length (bp)
GAPDH	F	GTC TTC ACT ACC ATG GAG AAG G	NM001034034	201
	R	TCA TGG ATG ACC TTG GCC AG		
YWHAZ	F	CAG GCT GAG CGA TAT GAT GAC	BC102382	141
	R	GAC CCT CCA AGA TGA CCT AC		
Ubiquitin	F	AGA TCC AGG ATA AGG AAG GCA T	NM174133	198
	R	GCT CCA CTT CCA GGG TGA T		
IL-1 β	F	AGT GCC TAC GCA CAT GTC TTC	NM174133	198
	R	TGC GTC ACA CAG AAA CTC GTC		
IL-6	F	GCT GAA TCT TCC AAA AAT GGA GG	NM173923	215
	R	GCT TCA GGA TCT GGA TCA GTG		
IL-8	F	ACA CAT TCC ACA CCT TTC CAC	AF232704	149
	R	ACC TTC TGC TCC CAC TTT TC		
TNF- α	F	CCA CGT TGT AGC CGA CAT C	NM173966	155
	R	CCC TGA AGA GGA CCT GTG AG		
TLR2	F	CAT TCC CTG GCA AGT GGA TTA TC	AY364629	201
	R	GGA ATG GCC TTC TTG TCA ATG G		
TLR4	F	TAT GAA CCA CTC CAC TCG CTC	DQ839566	207
	R	CAT CAT TTG CTC AGC TCC CAC		

¹Griesbeck-Zilch et al. (2008).²YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; TNF- α = tumor necrosis factor- α ; TLR = toll-like receptor.

genes: GAPDH, ubiquitin, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and the results were presented as $-\Delta\Delta C_T$ values (Livak and Schmittgen, 2001). The relative mRNA expression of the target gene at each time point was calculated using the following equation:

$$\Delta C_T = C_{T(\text{target gene})} - C_{T(\text{AM of housekeeping genes})};$$

$$\Delta\Delta C_T = \Delta C_{T(\text{time X})} - \Delta C_{T(\text{time 0})}.$$

Statistical Analysis

All statistical evaluations were conducted by use of the SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL). Data were analyzed by ANOVA for repeated measures. Differences between means were compared with a paired *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Clinical Signs After Challenge

All inoculated cows developed fever (>39.5°C) within the first 9 h, and maximal values (39.9 $\pm$ 0.4°C) occurred between 6 and 9 h after inoculation (Figure 1A). This was accompanied by a significant decrease in leukocyte counts 8 h after *E. coli* inoculation (Figure 1B). Daily milk production decreased by 33.6% (data not shown) on the first day after challenge, and the 6 cows remained significantly depressed until 6 d post-

inoculation (**PI**). Abnormal milk with clots or flakes from challenged quarters was present in all the cows during the second and third days after challenge. Milk SCC of uninfected quarters in the 6 cows was below 5×10^5 cells/mL throughout the study. Within 6 h of inoculation, milk SCC of *E. coli*-infected quarters rapidly exceeded 1×10^7 cells/mL (data not shown) in 3 of the cows, and remained at this level until 96 h PI, and then decreased to around 1×10^6 cells/mL at the end of the study at 168 h. Udder parameters, such as swelling and elevated quarter temperature, appeared from 6 h after inoculation in the infected quarters. The number of *E. coli* in the infected quarters peaked at 6 h after inoculation, followed by a rapid clearance within 12 h.

Cytokines, APP, and TLR in Blood

Serum IL-6 concentrations were significantly elevated from 4 h (*P* = 0.013) to 12 h (*P* = 0.022) and peaked at 6 h (*P* < 0.001) after inoculation, then gradually decreased to the preinoculation levels (Figure 2A). Serum TNF- α concentrations were significantly elevated from 4 (*P* = 0.023) to 12 h (*P* = 0.046), peaked at 6 h (*P* = 0.004) PI, and then gradually decreased to the preinoculation level (Figure 2B).

Serum SAA concentrations were significantly elevated from 6 (*P* < 0.001) to 12 h (*P* = 0.048) and peaked at 6 h after inoculation (Figure 2C). Serum Hp concentrations decreased significantly at 48 h (*P* = 0.05) and 168 h (*P* = 0.18) after inoculation compared with 0 h (Figure 2D). However, the concentrations of serum cortisol were unaltered (data not shown). Concentra-

Figure 1. Rectal temperature (A) and leukocytes in blood (B) in dairy cows following intramammary inoculation with *Escherichia coli*. Values are means of 6 observations with the standard error of means represented by vertical bars. Values differ significantly ($*P < 0.05$) from 0 h.

tions of serum sTLR2 and sTLR4 after inoculation did not differ relative to the preinoculation levels (Figure 3A and B).

mRNA Expression of TLR and Cytokines in Milk Somatic Cells

Intramammary inoculation of *E. coli* caused an increase in TLR2 mRNA expression (Figure 4A); expression increased significantly at 12 h ($P < 0.001$) and 24 h ($P < 0.05$) and decreased at 48 h and 72 h PI. However, TLR4 mRNA expression did not differ relative to the preinoculation level during the observed period (Figure 4B).

Expression of IL-1 β mRNA was significantly elevated at 6 h ($P < 0.01$) and 12 h ($P < 0.001$), and peaked at 12 h with a slight decrease at 48 h and 72 h PI (Figure 5A). Similarly, expression of TNF- α mRNA increased significantly at 12 h ($P < 0.01$) and 24 h ($P < 0.001$) PI (Figure 5D). Expression of IL-8 mRNA showed a significant increase at 12 h ($P < 0.05$) and 24 h ($P < 0.01$) and a decrease at 48 h and 72 h PI compared with the preinoculation level (Figure 5C). However, IL-6 mRNA expression decreased in the observation period and then gradually increased at 48 h PI (Figure 5B).

DISCUSSION

Intramammary infection with *E. coli* elicited a systemic response characterized by the induction of fever and a localized response characterized by proinflammatory cytokine production and elevated milk SCC. The clinical symptoms, metabolism changes, and APP and inflammatory cytokine profiles in blood and mam-

mary glands have been previously shown in cows with experimentally induced *E. coli* mastitis (Bannerman et al., 2004). At the present time, however, the role of TLR and soluble mediators of the early inflammatory response in the mammary gland of the cow is not known. The present study investigated TLR2 and TLR4 mRNA expression in milk somatic cells and their soluble forms sTLR2 and sTLR4 in blood during innate immune responses of dairy cows to experimentally induced *E. coli* mastitis.

Increases in the concentration of the proinflammatory cytokine TNF- α , a potent inducer of fever, in blood were observed from 4 to 12 h and peaked at 6 h after infection, a time that was temporally coincident with maximal increases in body temperature. Similarly, increases in serum IL-6 concentration were observed within 12 h of *E. coli* infection and were temporally coincident with increases in serum SAA concentrations. The induction of a systemic response reported here, as well as the temporal changes in Hp and leukocytes in blood, was consistent with previous studies on bovine mastitis (Eckersall et al., 2001; Bannerman et al., 2004; Gronlund et al., 2005).

Sensing the presence of bacteria in the lumen of the mammary gland is an important step in innate immunity. Milk somatic cells provide an important link between the external environment and the body interior because they are able to recognize conserved molecular patterns of invading microorganisms via pattern recognition receptors, including TLR (Rainard and Riollet, 2006). *Escherichia coli* is a species of gram-negative bacteria that is expected to upregulate only TLR4. Toll-like receptor 4 has been identified as the pattern recognition receptor for LPS that initiates the PAMP

Figure 2. Serum IL-6, tumor necrosis factor- α (TNF- α), serum amyloid A (SAA), and haptoglobin (Hp) concentrations in dairy cows following intramammary inoculation with *Escherichia coli*. Values differ significantly (* $P < 0.05$; ** $P < 0.01$) from 0 h.

from gram-negative bacteria (Akira and Takeda, 2004). However, a previous study has shown that expression of both TLR2 and TLR4 mRNA increased in mammary glands of cows infected with mastitis caused by either *Staph. aureus* or *E. coli* (Goldammer et al., 2004). It was somewhat unexpected that no increase in TLR4 mRNA expression was found in milk somatic cells collected from infected quarters in the current study. Our data are inconclusive regarding the effects of *E. coli* infection, as well as effects of normal lactation, on TLR4.

Interestingly, an increase in TLR2 mRNA expression was found in milk somatic cells collected from infected quarters of cows challenged with *E. coli*. This is in accordance with previous studies in which upregulated expression of TLR2 mRNA was observed in mammary glands challenged with LPS (Ibeagha-Awemu et al., 2008) as well as with *E. coli* (Yang et al., 2008). It has been shown that mRNA expression of TLR2 and TLR4

was higher in mammary epithelial cells collected from infected quarters of cows challenged with *E. coli* relative to those infected with *Staph. aureus* (Yang et al., 2008); this result suggests better pathogen recognition of gram-negative bacteria and more effective activation of the immune response, resulting in a severe but not chronic form of mastitis. Taken together, these findings may point to as-yet unknown factors in the mammary gland that would facilitate recognition of microbial products (whether from gram-negative or gram-positive bacteria) by both TLR4 and TLR2.

Although most TLR are transmembrane receptors, soluble forms of some sTLR have recently been identified in amniotic fluid and saliva in humans (Dulay et al., 2009; Zunt et al., 2009). The soluble form of TLR2, sTLR2, is produced by posttranslational modification of the anchored TLR2 that is constitutively expressed in blood monocytes and found in human plasma and

Figure 3. Serum soluble toll-like receptor (sTLR)2 (A) and sTLR4 (B) concentrations in dairy cows following intramammary inoculation with *Escherichia coli*. Values are means of 6 observations with the standard error of means represented by vertical bars.

milk (LeBouder et al., 2003). The soluble form of TLR4 is an alternative splice variant of TLR4 that was shown to be expressed as a mechanism to inhibit LPS-mediated TNF- α production and nuclear factor κ B activation (Iwami et al., 2000). Some sTLR are potential biomarkers for infectious disease in humans (Dulay et al., 2009). Plasma sTLR may originate from both monocytes and neutrophils (Kurt-Jones et al., 2002; LeBouder et al., 2003), and sTLR were proposed to be produced from the conversion of cell-surface TLR, which is consistent with conversion of CD14 into soluble CD14 (Durieux et al., 1994). This process involves endocytosis of the cell surface receptor and conversion into sTLR by processing in an internal acidic compartment and subsequent release by exocytosis. Cell activation resulted in a rapid

down-modulation of cell-surface TLR and a similar fast release of sTLR (LeBouder et al., 2003). Soluble TLR were thought to function as decoy receptors to sequester circulating PAMP (Srinivasan et al., 2008; Dulay et al., 2009; Zunt et al., 2009). Recombinant TLR attenuate TLR-mediated cellular activation (Iwami et al., 2000; LeBouder et al., 2003; Hyakushima et al., 2004). Relatively stable concentrations of sTLR2 (2.641 ± 0.22 ng/mL) and sTLR4 (2.94 ± 0.39 ng/mL) were detected in our study, but these results have not been verified by other recent studies in dairy cows. It was reported that cell sensitivity increases when the amount of sTLR in serum decreases, which suggests that serum sTLR may contribute to the regulation, and thus the efficiency, of the innate immune response to invading

Figure 4. Expression of toll-like receptor (TLR) 2 and TLR4 mRNA in milk somatic cells collected from dairy cows following intramammary inoculation with *Escherichia coli*. Data are means of 6 observations with the standard error of means represented by vertical bars. Values differ significantly (* $P < 0.05$; *** $P < 0.001$) from 0 h.

Figure 5. Expression of IL-1, IL-6, IL-8, and tumor necrosis factor- α (TNF- α) mRNA in milk somatic cells collected from dairy cows following intramammary inoculation with *Escherichia coli*. Data are means of 6 observations with the standard error of means represented by vertical bars. Values differ significantly (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) from 0 h.

pathogens (LeBouder et al., 2003). Relatively stable amounts of sTLR2 and sTLR4 reported in the present study suggest that sTLR2 and sTLR4 might protect the host by sequestering PAMP, thus avoiding excess proinflammatory response during *E. coli* mastitis.

During mastitis, changes in IL-1 β , IL-6, and TNF- α , as well as other cytokines such as IL-8, have been well characterized systemically and locally (Lee et al., 2006; Griesbeck-Zilch et al., 2008; Petzl et al., 2008). Systemically, these cytokines are known to induce an acute phase reaction associated with fever and production of APP, and they could be used as markers for subclinical coliform mastitis in cows (Nielsen et al., 2004). Locally, the source of these cytokines is mainly from resident cells within the mammary gland, including epithelial cells and macrophages. These cell types in the mammary gland could rapidly produce an array of cytokines and other inflammatory mediators that mediate inflammation, including IL-1 β , IL-6, TNF- α , and IL-8 (Al-

luwaimi et al., 2003). Furthermore, neutrophils, which are recruited to the mammary gland and constitute the majority of the somatic cells in the mammary gland during mastitis, could also release these cytokines (Lee et al., 2006; Baumert et al., 2009).

Many of the biological effects of IL-1 on host innate immune responses to infection are similar to those of TNF- α , including activation of endothelial cells and leukocytes and systemic induction of fever and APP synthesis (Dinarello, 1996b; Pruitt et al., 1995). Increases in IL-1 expression during bovine mastitis have been most frequently characterized in experimental *E. coli* intramammary infections. In the present study, an increase in IL-1 β mRNA expression was observed in milk somatic cells isolated from quarters infected with *E. coli*, in accordance with previous studies in which IL-1 β is upregulated at the mRNA level in cells isolated from cows with mastitis (Riollet et al., 2001; Peli et al., 2003) and in bovine mammary epithelial cells exposed

to *E. coli* or *E. coli*-derived products in vitro (McClenahan et al., 2005; Pareek et al., 2005; Strandberg et al., 2005; Lahouassa et al., 2007).

Tumor necrosis factor- α is a highly proinflammatory cytokine with both beneficial and injurious properties (van der Poll and Lowry, 1995). In accordance with our results, previous studies have shown that TNF- α increased at both the protein level in blood and mRNA level in milk somatic cells collected from cows with experimentally induced *E. coli* mastitis (Blum et al., 2000; Lee et al., 2006). Experimental infection with a lower inoculum (10^4 cfu/quarter) of *E. coli* has been reported to result in the detection of TNF- α concentrations between 0.1 and 10 ng/mL in blood (Hirvonen et al., 1999; Hoeben et al., 2000).

Interleukin-6 is a pleiotropic cytokine with both pro- and antiinflammatory properties. Among the clearly defined antiinflammatory properties of IL-6 is its ability to inhibit expression of IL-1 β and TNF- α and to stimulate expression of IL-1-receptor antagonist and soluble TNF receptor (Moshage, 1997; Papanicolaou et al., 1998). Although TNF- α and IL-1 β also induce hepatic APP synthesis, evidence exists that IL-6 induces the upregulation of a different set of APP (Moshage, 1997). Interestingly, an elevated concentration of serum IL-6 was detected, which is in accordance with previous studies in cows (Hagiwara et al., 2001). In the present study, however, IL-6 mRNA expression was markedly reduced in cells isolated from quarters infected with *E. coli*.

Interleukin-8 is chemotactic cytokine that is upregulated in response to infection (Kobayashi, 2008). Previous studies have shown that IL-8 mRNA expression increases in infected mammary glands compared with healthy glands during *E. coli* mastitis (Vangroenweghe et al., 2005; Lee et al., 2006). Similarly, an increase in IL-8 mRNA expression in milk somatic cells isolated from *E. coli*-infected glands was confirmed in our study.

CONCLUSIONS

To our knowledge, the present report is the first study to evaluate soluble forms of TLR2 and TLR4 in blood during *E. coli*-induced mastitis. We found that expression of TLR2 mRNA increased in milk somatic cells collected from infected quarters of cows challenged with *E. coli*, whereas the concentrations of sTLR2 and sTLR4 remained unchanged after challenge. Based on the present findings, we conclude that currently unknown factors in the mammary gland likely facilitate recognition of microbial products by both TLR4 and TLR2, and that sTLR2 and sTLR4 may protect the host by sequestering PAMP during *E. coli* mastitis.

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REFERENCES

- Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782–787.
- Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* 4:499–511.
- Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783–801.
- Alluwaime, A. M., C. M. Leutenegger, T. B. Farver, P. V. Rossitto, W. L. Smith, and J. S. Cullor. 2003. The cytokine markers in *Staphylococcus aureus* mastitis of bovine mammary gland. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 50:105–111.
- Bannerman, D. D., M. J. Paape, J. W. Lee, X. Zhao, J. C. Hope, and P. Rainard. 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin. Diagn. Lab. Immunol.* 11:463–472.
- Baumert, A., R. M. Bruckmaier, and O. Wellnitz. 2009. Cell population, viability, and some key immunomodulatory molecules in different milk somatic cell samples in dairy cows. *J. Dairy Res.* 76:356–364.
- Blum, J. W., H. Dosogne, D. Hoeben, F. Vangroenweghe, H. M. Hammon, R. M. Bruckmaier, and C. Burvenich. 2000. Tumor necrosis factor-alpha and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domest. Anim. Endocrinol.* 19:223–235.
- Dinarelo, C. A. 1996a. Biologic basis for interleukin-1 in disease. *Blood* 87:2095–2147.
- Dinarelo, C. A. 1996b. Cytokines as mediators in the pathogenesis of septic shock. *Curr. Top. Microbiol. Immunol.* 216:133–165.
- Dulay, A. T., C. S. Buhimschi, G. Zhao, E. A. Oliver, A. Mbele, S. Jing, and I. A. Buhimschi. 2009. Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. *J. Immunol.* 182:7244–7253.
- Durieux, J.-J., N. Vita, O. Popescu, F. Guette, J. Calzada-Wack, R. Munker, R. E. Schmidt, J. Lupker, P. Ferrara, H. W. Löms Ziegler-Heitbrock, and M. O. Labeta. 1994. The two soluble forms of the lipopolysaccharide receptor, CD14: Characterization and release by normal human monocytes. *Eur. J. Immunol.* 24:2006–2012.
- Eberhart, R. J. 1984. Coliform mastitis. *Vet. Clin. North Am. Large Anim. Pract.* 6:287–300.
- Eckersall, P. D., F. J. Young, C. McComb, C. J. Hogarth, S. Safi, A. Weber, T. McDonald, A. M. Nolan, and J. L. Fitzpatrick. 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Vet. Rec.* 148:35–41.
- Goldammer, T., H. Zerbe, A. Molenaar, H. J. Schuberth, R. M. Brunner, S. R. Kata, and H. M. Seyfert. 2004. Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. *Clin. Diagn. Lab. Immunol.* 11:174–185.
- Griesbeck-Zilch, B., H. H. Meyer, C. H. Kuhn, M. Schwerin, and O. Wellnitz. 2008. *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J. Dairy Sci.* 91:2215–2224.
- Grönlund, U., C. Hallen Sandgren, and K. Persson Waller. 2005. Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. *Vet. Res.* 36:191–198.
- Hagiwara, K., H. Yamanaka, K. Hisaeda, S. Taharaguchi, R. Kirisawa, and H. Iwai. 2001. Concentrations of IL-6 in serum and whey from healthy and mastitic cows. *Vet. Res. Commun.* 25:99–108.

- Hallman, M., M. Ramet, and R. A. Ezekowitz. 2001. Toll-like receptors as sensors of pathogens. *Pediatr. Res.* 50:315–321.
- Hirvonen, J., K. Eklund, A. M. Teppo, G. Huszenicza, M. Kulcsar, H. Saloniemi, and S. Pjörälä. 1999. Acute phase response in dairy cows with experimentally induced *Escherichia coli* mastitis. *Acta Vet. Scand.* 40:35–46.
- Hoeben, D., C. Burvenich, E. Trevisi, G. Bertoni, J. Hamann, R. M. Bruckmaier, and J. W. Blum. 2000. Role of endotoxin and TNF- α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. *J. Dairy Res.* 67:503–514.
- Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313–1318.
- Hyakushima, N., H. Mitsuzawa, C. Nishitani, H. Sano, K. Kuronuma, M. Konishi, T. Himi, K. Miyake, and Y. Kuroki. 2004. Interaction of soluble form of recombinant extracellular TLR4 domain with MD-2 enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. *J. Immunol.* 173:6949–6954.
- Ibeagha-Awemu, E. M., J. W. Lee, A. E. Ibeagha, D. D. Bannerman, M. J. Paape, and X. Zhao. 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. *Vet. Res.* 39:11.
- Iwami, K. I., T. Matsuguchi, A. Masuda, T. Kikuchi, T. Musikacharoen, and Y. Yoshikai. 2000. Cutting edge: Naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling. *J. Immunol.* 165:6682–6686.
- Kobayashi, Y. 2008. The role of chemokines in neutrophil biology. *Front. Biosci.* 13:2400–2407.
- Koj, A. 1996. Initiation of acute phase response and synthesis of cytokines. *Biochim. Biophys. Acta* 1317:84–94.
- Kurt-Jones, E. A., L. Mandell, C. Whitney, A. Padgett, K. Gosselin, P. E. Newburger, and R. W. Finberg. 2002. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. *Blood* 100:1860–1868.
- Lahouassa, H., E. Moussay, P. Rainard, and C. Riollot. 2007. Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*. *Cytokine* 38:12–21.
- LeBouder, E., J. E. Rey-Nores, N. K. Rushmere, M. Grigorov, S. D. Lawn, M. Affolter, G. E. Griffin, P. Ferrara, E. J. Schiffrin, B. P. Morgan, and M. O. Labeta. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. *J. Immunol.* 171:6680–6689.
- Lee, J. W., D. D. Bannerman, M. J. Paape, M. K. Huang, and X. Zhao. 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR. *Vet. Res.* 37:219–229.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods* 25:402–408.
- McClenahan, D. J., J. P. Sotos, and C. J. Czuprynski. 2005. Cytokine response of bovine mammary gland epithelial cells to *Escherichia coli*, coliform culture filtrate, or lipopolysaccharide. *Am. J. Vet. Res.* 66:1590–1597.
- Moshage, H. 1997. Cytokines and the hepatic acute phase response. *J. Pathol.* 181:257–266.
- Nielsen, B. H., S. Jacobsen, P. H. Andersen, T. A. Niewold, and P. M. Heegaard. 2004. Acute phase protein concentrations in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. *Vet. Rec.* 154:361–365.
- Papanicolaou, D. A., R. L. Wilder, S. C. Manolagas, and G. P. Chrousos. 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med.* 128:127–137.
- Pareek, R., O. Wellnitz, R. Van Dorp, J. Burton, and D. Kerr. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. *J. Appl. Genet.* 46:171–177.
- Peli, A., A. Scagliarini, D. Britti, and A. Boari. 2003. Detection of proinflammatory and regulatory cytokines in bovine milk using RT-PCR. *Vet. Res. Commun.* 27(Suppl. 1):779–781.
- Petzl, W., H. Zerbe, J. Gunther, W. Yang, H. M. Seyfert, G. Nurnberg, and H. J. Schuberth. 2008. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet. Res.* 39:18.
- Pruitt, J. H., E. M. Copeland, and L. L. Moldawer. 1995. Interleukin-1 and interleukin-1 antagonism in sepsis, systemic inflammatory response syndrome, and septic shock. *Shock* 3:235–251.
- Rainard, P., and C. Riollot. 2006. Innate immunity of the bovine mammary gland. *Vet. Res.* 37:369–400.
- Riollot, C., P. Rainard, and B. Poutrel. 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. *J. Dairy Sci.* 84:1077–1084.
- Seegers, H. C., V. C. Hood, B. L. Kidd, S. C. Cruwys, and D. A. Walsh. 2003. Enhancement of angiogenesis by endogenous substance P release and neurokinin-1 receptors during neurogenic inflammation. *J. Pharmacol. Exp. Ther.* 306:8–12.
- Srinivasan, M., K. N. Kodumudi, and S. L. Zunt. 2008. Soluble CD14 and toll-like receptor-2 are potential salivary biomarkers for oral lichen planus and burning mouth syndrome. *Clin. Immunol.* 126:31–37.
- Strandberg, Y., C. Gray, T. Vuocolo, L. Donaldson, M. Broadway, and R. Tellam. 2005. Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* 31:72–86.
- Suffredini, A. F., G. Fantuzzi, R. Badolato, J. J. Oppenheim, and N. P. O'Grady. 1999. New insights into the biology of the acute phase response. *J. Clin. Immunol.* 19:203–214.
- Thijs, L. G., A. B. Groeneveld, and C. E. Hack. 1996. Multiple organ failure in septic shock. *Curr. Top. Microbiol. Immunol.* 216:209–237.
- Uthaisangsook, S., N. K. Day, S. L. Bahna, R. A. Good, and S. Hara-guchi. 2002. Innate immunity and its role against infections. *Ann. Allergy Asthma Immunol.* 88:253–264.
- van der Poll, T., and S. F. Lowry. 1995. Tumor necrosis factor in sepsis: Mediator of multiple organ failure or essential part of host defense? *Shock* 3:1–12.
- Vangroenweghe, F., L. Duchateau, P. Boutet, P. Lekeux, P. Rainard, M. J. Paape, and C. Burvenich. 2005. Effect of carprofen treatment following experimentally induced *Escherichia coli* mastitis in primiparous cows. *J. Dairy Sci.* 88:2361–2376.
- Vangroenweghe, F., L. Duchateau, and C. Burvenich. 2004. Moderate inflammatory reaction during experimental *Escherichia coli* mastitis in primiparous cows. *J. Dairy Sci.* 87:886–895.
- Werling, D., J. Piercy, and T. J. Coffey. 2006. Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells-potential role in pathogen discrimination? *Vet. Immunol. Immunopathol.* 112:2–11.
- Wilson, D. J., R. N. Gonzalez, and H. H. Das. 1997. Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. *J. Dairy Sci.* 80:2592–2598.
- Yang, W., H. Zerbe, W. Petzl, R. M. Brunner, J. Gunther, C. Draing, S. von Aulock, H. J. Schuberth, and H. M. Seyfert. 2008. Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (CXCL8) expression in the udder. *Mol. Immunol.* 45:1385–1397.
- Zhen, Y. H., L. J. Jin, J. Guo, X. Y. Li, Y. N. Lu, J. Chen, and Y. P. Xu. 2008. Characterization of specific egg yolk immunoglobulin (IgY) against mastitis-causing *Escherichia coli*. *Vet. Microbiol.* 130:126–133.
- Zunt, S. L., L. V. Burton, L. I. Goldblatt, E. E. Dobbins, and M. Srinivasan. 2009. Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells. *Clin. Exp. Immunol.* 156:285–293.