Differential gene expression of the toll-like receptor-4 cascade and neutrophil function in early- and mid-lactating dairy cows

M. G. H. Stevens,* L. J. Peelman,† B. De Spiegeleer,‡ A. Pezeshki,* G. R. Van De Walle,* L. Duchateau,* and C. Burvenich*¹

*Department of Comparative Physiology and Biometrics, Faculty of Veterinary Medicine, and

†Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, B-9820 Merelbeke, Belgium

‡Drug Quality and Registration (DruQuaR) group, Faculty of Pharmaceutical Sciences, Ghent University, B-9000 Ghent, Belgium

ABSTRACT

During early lactation, neutrophils display several reduced immune functions. Particularly, a delayed recruitment of neutrophils into the infected udder seems to be one of the underlying events involved in the severity of postpartum *Escherichia coli* intramammary infections. The purpose of this study was to analyze the effect of in vitro chemotaxis and diapedesis on the expression of toll-like receptor-4 (TLR4)-related genes in bovine blood neutrophils isolated from 10 early-lactating (EL) and 10 mid-lactating (ML) cows. Functional characterization of the neutrophil population was performed by measuring phagocytosis and production of reactive oxygen species (chemiluminescence). Messenger RNA was extracted from neutrophils, and the expression of TLR4 and associated genes in EL and ML cows was analyzed by reverse-transcription quantitative PCR. To study the effect of chemotaxis and diapedesis on the expression of genes of the TLR4 cascade, neutrophils were stimulated to (trans)migrate in response to C5a using in vitro models. Our salient findings were that both neutrophil migration in vitro and lactation stage induced significant changes in the expression of several genes of the TLR4 signaling cascade. Before migration, expression of *TRAF6*, *ATF3*, *RELA*, *IL8*, and *C5aR* were lower in EL than in ML cows. Diapedesis and chemotaxis induced an increase in expression of *TLR4*, *ATF3*, and *IL8* in both EL and ML cows. Diapedesis resulted in a downregulation of *Syk*, a TLR4-associated gene, in ML cows. This study shows that the perturbations in neutrophil functions during EL are accompanied by modulation of TLR4 pathway genes. These data can contribute to the understanding of the mechanisms explaining the relationship between stage of lactation and risk of severe *E. coli* mastitis.

Key words: diapedesis, chemotaxis, toll-like receptor-4 (TLR4), neutrophil

INTRODUCTION

The innate immune system is the most important defense mechanism in the bovine mammary gland. When pathogens develop in the udder cisterns in sufficient numbers, an inflammatory response is activated. Inflammation starts with the migration of neutrophils toward the site of infection and involves a 2-step process of diapedesis and chemotaxis. Neutrophil migration is one of the key phenomena during the inflammatory process and dysfunction can lead to chronic and even fatal infections such as mastitis in dairy cattle (Burvenich et al., 1994).

Mastitis, an inflammation of the mammary gland, has important effects on both animal health and economic loss in the dairy industry. The severity of symptoms during clinical *Escherichia coli* mastitis, reflecting the involvement of sepsis, is mainly determined by host factors (Burvenich et al., 2003). In particular, lactation stage and parity of the cow have important effects on mastitis severity. Around parturition, cows are more susceptible to the development of severe mastitis, which is partly explained by the fact that these cows have poor neutrophil recruitment to the udder (Vangroenweghe et al., 2005). Cows in mid lactation show a rapid influx of neutrophils into the infected mammary gland. This massive influx of neutrophils results in a mild to moderate inflammatory response and fast curing of the infection. Thus, neutrophil migration to the site of infection seems a very important factor for the innate immune response.

The involvement of the toll-like receptor (TLR)-4 pathway in the development of bovine *E. coli* mastitis has been hypothesized by De Schepper et al. (2008). When gram-negative bacteria such as *E. coli* enter the mammary gland, they shed LPS. Neutrophils recognize this pathogen-associated molecular pattern (PAMP) through TLR4 (Beutler et al., 2001).

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¹Corresponding author: christian.burvenich@ugent.be

However, TLR4 does not recognize LPS by itself. It requires the help of CD14 and myeloid differentiation protein 2 (**MD2**; Fitzgerald et al., 2004). The binding of LPS to the TLR4/CD14/MD2 receptor complex activates downstream signaling, which can occur via an adaptor protein myeloid differentiation factor 88 (**MyD88**)-dependent or a MyD88-independent pathway [also known as the TIR-containing adaptor molecule-1 (**TICAM-1**)-dependent pathway; Akira and Hoshino, 2003]. Signaling via MyD88 subsequently results in the activation of the tumor necrosis factor- α receptor-associated factor-6 (**TRAF-6**), which forms a complex with mitogen-activated protein kinase kinase 7 (**MAP3K7**). This leads to the activation of a kinase complex that consists of α (**IKK α**) and β subunits (Akira and Hoshino, 2003). Eventually, the nuclear factor- κ B (**NF- κ B**) complex is translocated to the nucleus and the transcription of proinflammatory genes (e.g., *IL8*) is initiated. The NF- κ B complex consists of several subunits that are members of a family of structurally related proteins. Five different Rel proteins (also called Rel/NF- κ B proteins) have been identified so far: p50, p52, p65 (also called **RELA**), RelB, and c-Rel (Ghosh et al., 1998).

Toll-like receptor-4 signaling is strictly regulated by endogenous inhibitory molecules that act at different levels of the signaling cascade. The E3 ubiquitin-protein ligase (**Triad3A**) interacts with the toll/IL-1 receptor domain of TLR and promotes their proteolytic degradation (Chuang and Ulevitch, 2004). Phosphatidylinositol 3-kinase (**PI3K**) regulates TLR4 signaling at the plasma membrane, and PI3K activation leads to a depletion of PIP₂ stores at the membrane, resulting in the dissociation of TIRAP, which forms a bridge between TLR4 and MyD88 and thereby, diminishes TLR4-dependent signaling (Laird et al., 2009). In macrophages it was observed that the activating transcription factor 3 (**ATF-3**) inhibited *IL6* and *IL12b* transcription by altering chromatin structure, thereby restricting access to transcription factors. It was also shown that ATF3 itself is induced by LPS and it was concluded that ATF3 regulates a TLR4-dependent inflammatory response in a negative-feedback loop (Gilchrist et al., 2006).

Spleen tyrosine kinase (**Syk**) is a nonreceptor tyrosine kinase most abundant in cells of the hematopoietic lineages (Sada et al., 2001); Syk is involved in a variety of biological functions such as cell adhesion, innate pathogen recognition, platelet activation, vascular development, and osteoclast maturation (see review by Mócsai et al., 2010). It is usually not shown in the traditional presentation of the TLR4 cascade but it has been reported that Syk is associated with the TLR4 receptor (Arndt et al., 2004; Chaudhary et al., 2007).

We hypothesized that the diapedesis process itself might alter the TLR4 signaling pathway in such a way that neutrophils from cows in early lactation are not able to induce an adequate TLR4-mediated innate response. To our knowledge nothing is known yet about the effect of neutrophil migration on TLR4 signaling in neutrophils. Therefore, this preliminary study was done to analyze the effect of diapedesis and chemotaxis on the expression of members of the TLR4 signaling cascade in neutrophils from cows in early and mid lactation.

MATERIALS AND METHODS

Animals

Ten Holstein-Friesian cows 3 d postpartum (early lactation, **EL**) and 10 cows 149 to 151 d postpartum (mid lactation, **ML**) were used. Cows were housed at the dairy farm (Biocenter Agri-Vet, Melle, Belgium) of Ghent University, and all animals were clinically healthy. Milk production ranged between 6,461 and 14,147 kg/yr with percentages of fat and protein in milk of 4.23 and 3.43, respectively. Somatic cell count was <200,000 cells/mL for all cows. Cows were milked with a milk robot (DeLaval VMS, Drongen, Belgium) at an average voluntary milking frequency of 2.7/d. Cow data (e.g., last milking time, milk yield, food quantity) were measured through a transmitter in the collar of each cow and uploaded into a computer. All cows had access to a TMR during the day. Approval from the local ethical committee (Ghent University) was obtained before commencement of the study (EC2008/108).

Study Design

Figure 1 shows an overview of the experimental study design. First, neutrophils from EL and ML cows were isolated and functionally characterized by measuring phagocytosis, oxidative burst, diapedesis (**DIA**), and chemotaxis (**CTX**) capacity, as described below. Based on these assays, neutrophils from EL and ML cows were characterized as immune-compromised and immune-competent, respectively. Then, RNA was isolated from unstimulated neutrophils in both groups and the expression levels of important genes of the TLR4 pathway were determined by reverse-transcription quantitative PCR (**RT-qPCR**). To analyze the effect of DIA on the TLR4 pathway, neutrophils were allowed to perform DIA through an epithelial cell layer in response to C5a. After DIA, neutrophils that performed DIA were quantified and RNA was isolated. The expression of the selected genes was measured by RT-qPCR and

compared with that of unstimulated neutrophils before migration. The effect of CTX on the TLR4 pathway in neutrophils from EL and ML cows was also studied. Therefore, neutrophils were allowed to migrate through a porous membrane in response to a C5a gradient. After migration, cells were quantified and RNA was isolated for expression analysis. Because C5a was used as a chemoattractant in the DIA and CTX assays, the expression of the C5a receptor (**C5aR**) was analyzed (Figure 1).

Blood Sampling and Neutrophil Purification

Blood samples for neutrophil function determination were collected aseptically via the coccygeal vein from EL and ML cows as described by Sears et al. (1978). Fifty-milliliter blood samples from each cow were collected in Vacutainer tubes (Becton Dickinson, Erembodegem, Belgium) containing 150 μ L of EDTA as anticoagulant. Blood was processed within 0.5 h of collection. Isolation of neutrophils was performed according to Siemens et

Figure 1. Schematic overview of the study design. After isolation from early- (EL) and mid- (ML) lactating cows, blood polymorphonuclear neutrophils were characterized by chemiluminescence and phagocytosis capacity. mRNA expressions from genes in the TLR4 pathway and associated genes were measured by reverse transcription-quantitative PCR before and after chemotaxis (CTX) or diapedesis (DIA) through in vitro systems. REST = unstimulated cells.

al. (2007). Briefly, red blood cells were lysed by adding 50 mL of RNase-free purified water. Five milliliters of 10% NaCl was added and the sample was centrifuged at $585 \times g$ for 10 min. Supernatant was discarded and the pellet was resuspended in Hanks' buffered salt solution (HBSS). Ten milliliters of the leukocyte suspension was layered on top of the Histopaque 1077/1119 gradient (Sigma-Aldrich, Bornem, Belgium) and centrifuged at $440 \times g$ for 25 min. After removal of the supernatant, neutrophils were washed by adding 50 mL of HBSS and centrifuged at $585 \times g$ for 10 min. All centrifugation steps occurred at room temperature (RT, 20°C). The final pellet was resuspended in 1 mL of PBS and stored on ice until used.

Cell viability was assessed with the Trypan blue exclusion test. Therefore, 10 μ L of the neutrophil suspension was transferred to a microcentrifuge tube and 590 μ L of a 0.4% Trypan blue solution (Sigma-Aldrich) was added. Cell counting was done in triplicate using a Bürker chamber. To determine purity, neutrophils were differentiated in smears and stained with Sangodiff G (Merck, Darmstadt, Germany). Slides were analyzed by bright-field microscopy, with a 100 $\times$ objective and immersion oil (Axioskop 2 MOT, Carl Zeiss N.V., Zaventem, Belgium).

Neutrophil Function Assays

Reagents. Ham's F12, RPMI-1640, and NCTC 135 media, and fetal bovine serum, lactose, lactalbumin hydrolysate, reduced L-glutathione, L-ascorbic acid, hydrocortisone, bovine insulin, antibiotic-antimycotic solution, 0.1% calfskin collagen (type I) solution, HBSS (Ca^{2+} and Mg^{2+}), phorbol 12-myristate 13-acetate (PMA), luminol, and endotoxin-free and ultrafiltered water were purchased from Sigma-Aldrich. Bovine serum albumin was purchased from Roche Diagnostics (Vilvoorde, Belgium).

Phagocytosis Assay. The Vybrant Phagocytosis Assay kit (Invitrogen, Merelbeke, Belgium) was used for nonopsonized phagocytosis of *E. coli* according to Siemens et al. (2007) with some modifications for the fluorescence microscopy analysis. In this study, 0.5 mL of RPMI-1640 was added to 1 mL of fluorescein isothiocyanate (FITC) to label the chemically killed *E. coli* (K-12 strain). Prior to use, the *E. coli* suspension was incubated for 30 min in a shaking water bath (37°C). One million neutrophils were suspended in 1 mL of RPMI-1640 (37°C). One hundred fifty microliters of RPMI-1640, 100 μ L of neutrophils, and 10 μ L of *E. coli* suspension were aliquoted in 1.5-mL polypropylene microcentrifuge tubes. The reaction was done in triplicate. The final neutrophil:*E. coli* ratio was 1:20. Neutrophils and *E. coli* were incubated for 20 min at

37°C and 5% CO_2 . The reaction was stopped by adding 4% cold paraformaldehyde. Trypan blue was used to quench extracellular fluorescence and to distinguish attached from ingested bacteria. Phagocytosis was examined with a fluorescence microscopy and phase contrast. For fluorescence, a 100 $\times$ immersion oil lens and a FITC filter set (excitation: BP485/20; emission: BP515-565) was used. One hundred neutrophils were counted per sample and the following parameters were calculated: mean number of particles per neutrophil, the percentage of neutrophils that performed phagocytosis, and the phagocytotic index, which was calculated using the following formula:

$$\left(\frac{N_{bac}}{N_{neutrophils}} \right) \times \%NP,$$

where N_{bac} = total number of bacteria; $N_{neutrophils}$ = total number of neutrophils; and $\%NP$ = percentage of neutrophils containing at least 1 bacterium.

Chemiluminescence Assay. Neutrophils were stimulated with or without PMA and luminol was used as luminescent agent as described by Mehrzad et al. (2001). Briefly, 400,000 neutrophils were suspended in Dulbecco's PBS and transferred to a 96-well plate. Luminol (0.30 mol/L) and PMA (200 ng/mL) were added, and PMA was replaced by Dulbecco's PBS for CL measurements from unstimulated neutrophils. All reactions were performed in duplicate. Results were included if CV was <10%. Chemiluminescence was measured with a luminometer (type LB96P, EG&G Berthold, Bad Wildbad, Germany). The area under the curve (AUC) was calculated over a period of 30 min and expressed as relative light units (RLU). The CL response was corrected for the actual number of viable neutrophils in each sample (Heyneman et al., 1990; Hoeben et al., 2000).

DIA Assay. Bovine mammary epithelial cells (BME-UV) were cultured in 75-cm² tissue culture treated, vented flasks at 37°C in 5% CO_2 . Cells were subcultured once every 3 to 5 d when flasks were approximately 80% confluent. Then, 3×10^5 BME-UV cells were added to Millicell-PCF inserts (12-mm diameter, 8.0- μ m pore size; Millipore, Bedford, MA) coated with 0.10% calfskin collagen type I. Models were cultured as described by Smits et al. (1996). The confluence of the epithelial monolayers was determined by epithelial resistance ($\Omega \cdot \text{cm}^2$) as follows: collagen-coated inserts without cells were used to determine baseline resistance and monolayer resistance was corrected using the following formula: $(R_{\text{measured}} - R_{\text{baseline}}) \times 0.60 \text{ cm}^2$ (R, Ω). In general, monolayers had an average resistance

of $96.65 \pm 4.11 \Omega\text{-cm}^2$ at d 14. Before use, cultures were washed with HBSS (containing Ca^{2+} and Mg^{2+}) to remove the culture medium and incubated for 30 min with HBSS at 37°C and 5% CO_2 . Next, the cultures were transferred to a 24-well tissue culture dish containing 600 μL of HBSS with 0.1% BSA and 50 ng/mL of bovine purified complement factor C5a (courtesy of P. Rainard, INRA UR1282 Laboratoire d'Infectiologie Animale et Santé Publique, Nouzilly, France). Then, 20×10^6 neutrophils were suspended in preheated HBSS with Ca^{2+} and Mg^{2+} (37°C) and added at the basal side of the epithelial cell monolayer. Neutrophils were allowed to perform diapedesis during 90 min at 37°C and 5% CO_2 . Transmigrated neutrophils were counted by using a hemocytometer.

CTX Assay. Millicell-PCF inserts (12-mm diameter, 8.0- μm pore size; Millipore) were incubated for 30 min with HBSS at 37°C and 5% CO_2 and transferred to a 24-well tissue culture dish containing 600 μL of HBSS with 0.1% BSA and 50 ng/mL of bovine purified complement factor C5a. Then, 20×10^6 neutrophils were suspended in preheated HBSS with Ca^{2+} and Mg^{2+} (37°C) and added at the apical chamber of the filter. Neutrophils were allowed to migrate through the filter for 90 min at 37°C and 5% CO_2 , and migrated neutrophils were counted with a hemocytometer.

TLR4 Signaling Cascade Before and After CTX and DIA

The expression of genes of the TLR4 pathway was determined in neutrophils of EL and ML cows with emphasis on the effect of diapedesis and chemotaxis.

RNA Isolation and cDNA Synthesis. Total RNA was extracted from the neutrophils using Total RNA Isolation Reagent (ABgene, Epsom, Surrey, UK), according to the manufacturer's protocol. The obtained RNA was dissolved in 15 μL of preheated MilliQ water (65°C) buffered with Tris-HCl (pH 8.0) (VWR International, Leuven, Belgium). The RNA concentration of the samples was measured with the Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Sint-Pieters-Leeuw, Belgium), and was between 0.70 and 63.42 ng/ μL . The quality of RNA was assessed by evaluation of the 28S and the 18S ribosomal bands on a 0.8% agarose gel. To remove genomic DNA, digestion was carried out by adding 4 μL of RQ1 RNase-free DNase (Promega, Leiden, the Netherlands) and 2 μL of RQ1 DNase Reaction Buffer (Promega) to 14 μL RNA, followed by spin-column purification (Microcon YM-100, Millipore, Brussels, Belgium). To assure that DNase treatment was successful, a minus RT control PCR was performed on the iCycler (Bio-Rad) in a total volume of 15 μL containing $2\times$ KAPA SYBR FAST qPCR

Master Mix Bio-Rad iCycler (KAPA Biosystems, Woburn, MA), GAPDH primers (Table 1) and 2.5 μL of template (RNA, genomic DNA as positive control, and water as negative control). The qPCR reaction consisted of a 3-min enzyme activation step at 95°C , followed by 40 amplification cycles (denaturation for 3 s at 95°C and annealing/elongation/SYBR detection for 30 s at 60°C) and a melt curve analysis (from 75 to 90°C with 0.3°C increments of 10 s). The amplification products were additionally analyzed on a 2% agarose gel (results not shown). The DNase-treated RNA was used for generating single-stranded cDNA by means of the Improm II reverse transcription system (Promega) using Random and Oligo dT primers (each 0.80 μL per reaction). The conversion was confirmed by a PCR with *GADPH* primers (giving an amplicon of different lengths on gDNA and cDNA) on $10\times$ diluted cDNA. All PCR assays included a positive control and a no-template control.

Reference Gene Selection and Primer Design. Reference genes used for normalization of the data are shown in Table 1 (De Ketelaere et al., 2006). To assess the stability of 6 selected candidate reference genes, their cycle threshold (*Ct*) values were measured on 5×10^6 neutrophils ($10\times$ diluted). The reactions were performed in duplicate in 2 independent runs. A correction for technical inter-run variation was carried out as described by Vandesompele et al. (2002). After calibration, the average *Ct* value of each sample was converted to raw data (not normalized quantitative data) using the measured gene-specific PCR efficiencies, and the stability of the different reference genes was assessed with the geNorm software (Vandesompele et al., 2002). After identification of the most stable reference genes, *Ct* values and raw data for these genes were measured and calculated on all EL and ML samples, as described previously. The primers for the TLR4 receptor and associated target genes were based on the mRNA sequences of the bovine genes (<http://www.ncbi.nlm.nih.gov>), and the primers were designed using the Primer3 software (http://www.sk.emblnet.org/cgi-bin/primer3_www.cgi). The optimal annealing temperature was set at 60°C , the optimal GC content at 50%, and the maximal self-complementarity was kept as low as possible. The presence of secondary structures in the amplicon was checked using 'mfold' (<http://www.bioinfo.rpi.edu/applications/mfold/dna>), and the specificity was checked using the Basic Local Alignment Search Tool at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). A list of the target genes and primer conditions is shown in Table 2.

RT-qPCR. The primer concentrations used were 2 μM for *GADPH* and *HPRT1* and 5 μM for all other primers. During each run, a $5\times$ dilution series was in-

Table 1. Primers of the reference genes used for normalization of the reverse transcription-quantitative PCR data

Gene	GenBank accession no.	Species	Sequence	Product size (bp)	T _a ¹ (°C)
<i>ACTB</i>	AY141970	Cow	5'-CCTCACGGAACGTGGTTACA-3' 5'-TCCTTGATGTCACGCACAATTT-3'	87	58
<i>GAPDH</i>	XM 618013	Cow	5'-TTCAACGGCACAGTCAAGG-3' 5'-ACATACTCAGCACCAGCATCAC-3'	119	60
<i>Histone H2A</i>	U62674	Mouse	5'-GTCTGGCAAGCAAGGAG-3' 5'-GATCTCGGCCGTTAGGTACTC-3'	182	60
<i>HPRTI</i>	AF176419	Cow	5'-TGCTGAGGATTTGGAGAAGG-3' 5'-CAACAGGTCGGCAAAGAAGT-3'	154	58
<i>YWHAZ</i>	BM446307	Cow	5'-GCATCCCACAGACTATTTCC-3' 5'-GCAAAGACAATGACAGACCA-3'	120	60
<i>18S rRNA</i>	AF176811	Cow	5'-AGAAACGGCTACCACATCCA-3' 5'-CACCAGACTTGCCCTCCA-3'	169	62

¹T_a = annealing temperature.

cluded to create a relative standard curve, which was used for PCR efficiency calculations. A no-template control was also added to every run. Absence of primer dimers was verified by melting curve analysis and by 2% gel agarose electrophoresis. The efficiencies were between 85.30 and 110.00%, and primer dimers were absent in all reactions.

Gene Expression Measurement and Data Analysis.

Cycle threshold values and raw expression

data were determined for the reference genes and target genes on all samples (10× diluted). Next, obtained relative quantities were normalized by dividing the raw expression data of the target genes by the normalization factor, which is the geometric mean of the raw expression data of the 4 most stable reference genes. For all statistical analyses, these normalized expression data were converted to logarithmic values (Vandesompele et al. 2002). Data are presented as means ±

Table 2. Primers of the toll-like receptor-4 (TLR4) receptor and associated genes for reverse transcription-quantitative PCR analysis

Gene	GenBank accession no.	Species	Sequence	Product size (bp)	T _a ¹ (°C)
<i>ATF3</i>	NM_001046193	Cow	5'-ACTGGTGTTTGAGGATTTTGCT-3' 5'-GCAGGCACTCTGTCTTCTCC-3'	260	64.0
<i>CD14</i>	NM_174008	Cow	5'-GAAGAGAGGGAAGAGGAATGAATT-3' 5'-TAATAAGGATGGGGCAGAAGG-3'	100	57.9
<i>C5aR</i>	NM_001007810	Cow	5'-ACTGGTCCTTTGGTGATGCT-3' 5'-GTGGGAAGTGGTCTATGTGG-3'	249	60.0
<i>IKKα</i>	XM_863907	Cow	5'-TCTCGTATTGAGCGGGAAAC-3' 5'-TCTGGAAGCAAATGGTCCT-3'	189	60.0
<i>IL8</i>	NM_173925	Cow	5'-CTGCCTAAACCCCAAGGAA-3' 5'-AACCCCTACACCAGACCCACA-3'	206	60.0
<i>MAP3K7</i>	NM_001081595	Cow	5'-CGCTTCTTCTTCTTCCTCGT-3' 5'-GCTCCTCTTCCAACAACCTC-3'	120	59.0
<i>MD2</i>	XM_864835	Cow	5'-AGCAGATTGGGTTCTCTTTTG-3' 5'-AAATGATGTCACTCGGGTAGG-3'	154	58.7
<i>MYD88</i>	NM_001014382	Cow	5'-CGCACAGACATACAGGCATC-3' 5'-GGTCCCAGTGAAAAAGGAAAA-3'	153	63.0
<i>PI3K</i>	NM_174574	Cow	5'-GTTACCCAAGAAGCAGAAAGG-3' 5'-ATGCCGATAGCAAAACCAA-3'	152	59.0
<i>RELA</i>	NM_001080242	Cow	5'-GCATCCAGACCAACAACAAC-3' 5'-CTCCAGAGTTCCGATTAC-3'	217	60.0
<i>SYK</i>	NM_001037465	Cow	5'-ACAAGGACAAAAGCGGGAAG-3' 5'-TGGCTCGTAAGGGTTGAATG-3'	248	61.0
<i>TICAM-1</i>	NM_001030301	Cow	5'-GGAGTCGTCCGAGCAGAAA-3' 5'-AGGATGATGAATGCCGAGTG-3'	201	61.0
<i>TLR4</i>	NM_174198	Cow	5'-CAGGGCAGGGAACAGTCAA-3' 5'-AGGAAAAGTGAGCCAAGACC-3'	203	59.0
<i>TRAF6</i>	NM_001034661	Cow	5'-TGAAATCGCAAGAAGAGGAGA-3' 5'-TGCATCGTGTGGACAAAGAG-3'	154	60.0
<i>TRIAD3A</i>	XM_883230	Cow	5'-GTGTTCCCGATGCTCTCT-3' 5'-CGATGCGTTTGAAGGTGTT-3'	125	62.1

¹T_a = annealing temperature.

Figure 2. Neutrophil functions in early- (EL) and mid- (ML) lactating cows. Neutrophils were collected from 10 cows sampled 3 d postpartum (□) and 10 cows sampled 149 to 151 d postpartum (■). A) Chemiluminescence (CL); B) mean percentage phagocytosis; and C) diapedesis and chemotaxis. AUC = area under the curve. Asterisks denote a significant difference ($P < 0.05$) between the EL and ML cows.

SEM. To compare the 2 periods (EL and ML) with respect to different response variables [CTX, DIA, and unstimulated (**REST**)], a mixed model with lactation stage and their interactions as the fixed effects and cow as the random effect was used to study the relationship between stage of lactation and gene expressions before and after CTX and DIA. A fixed effect model with lactation stage as fixed effect was used to study the relationship between lactation stage and neutrophil function. Hypothesis testing was done at 5% significance level. The normalized expression values (ΔCt) of the target genes were compared (1) in each lactation stage between resting and migrated neutrophils and (2) between EL and ML by calculating $\Delta\Delta Ct$. The $\Delta\Delta Ct$ was used to calculate the fold change by performing a $2^{-\Delta\Delta Ct}$ calculation.

RESULTS

Neutrophil Functions in EL Cows

To quantify the expected impairment of neutrophil function in EL cows, the ability of isolated neutrophils to perform phagocytosis, oxidative burst, diapedesis, and chemotaxis was measured. Before this functional testing, a differential count of the isolated cells was performed and showed that $94.80\% \pm 0.50$ of the isolated cells from EL cows and $94.20\% \pm 0.70$ of the cells from ML cows had a neutrophilic phenotype. In the neutrophil population of EL cows, $86.90\% \pm 1.20$ and in ML cows, $90.10\% \pm 1.60$ were mature segmented neutrophils. All neutrophils had a viability of $>99\%$.

Oxidative Burst. To measure the oxidative burst capacity of the neutrophils, the amount of reactive oxygen species (**ROS**) released by the neutrophils was calculated as the AUC. Unstimulated neutrophils from EL cows had a lower response compared with those of ML cows with an AUC of 6.86×10^6 and 8.48×10^6 RLU, respectively (Figure 2A). Stimulation of neutrophils with PMA increased the luminol-dependent response up to 8.27×10^7 for neutrophils from EL cows and 9.35×10^7 for neutrophils isolated from ML cows, which points toward a tendency for lower ROS production during EL, although this could not be confirmed statistically (Figure 2A).

Phagocytosis. The percentage of neutrophils performing phagocytosis of FITC-coated *E. coli* was 26% during EL and 32% during ML (Figure 2B; $P < 0.05$). In addition, neutrophils from EL cows contained, on average, 1.32 ± 0.04 bacteria, and those from ML cows contained 1.37 ± 0.04 bacteria (determined by phase contrast microscopy; $P > 0.05$). The phagocytotic index was lower in EL cows compared with ML cows (9.49 ± 1.30 vs. 14.60 ± 1.30 ; $P < 0.05$).

DIA. Neutrophils added to the basal chamber of the DIA model started to transmigrate in response to C5a added at the apical chamber. After 90 min, 7.29×10^5 EL neutrophils were counted in the basal chamber, whereas 1.24×10^6 ML neutrophils had performed DIA at that time point (Figure 2C; $P < 0.05$), indicating that neutrophil recruitment was lower in EL cows.

CTX. To quantify the efficiency of CTX, neutrophils were allowed to migrate through a porous membrane (12-mm diameter, 8.0- μ m pore size) in response to 50 ng/mL C5a. After 90 min, the neutrophils from ML

cows were significantly more numerous in the lower chamber of the CTX model compared with the neutrophils from EL cows (9.10×10^5 vs. 3.12×10^6 ; $P < 0.05$), indicating that neutrophils from ML cows migrated faster under influence of a chemotactic gradient than did neutrophils from EL cows (Figure 2C).

Based on all these data, namely the fact that the neutrophil population isolated from the EL cows had a lower ability to perform phagocytosis, DIA, and CTX, these neutrophils were considered as immune-compromised neutrophils. In contrast, neutrophils from

Table 3. Effect of lactation stage and migration on the mRNA expression of the toll-like receptor-4 (TLR4) pathway and associated genes in bovine neutrophils

Gene	Neutrophil group ¹	Early lactating (n = 10)	Mid lactating (n = 10)
<i>ATF3</i>	REST	-1.062 (0.200)	-0.617 (0.062)
	DIA	0.320 ^a (0.071)	0.043 ^a (0.114)
	CTX	0.183 ^a (0.112)	0.281 ^a (0.074)
<i>CD14</i>	REST	-0.044 (0.093)	-0.257 (0.124)
	DIA	0.222 (0.137)	0.473 ^a (0.129)
	CTX	0.233 (0.138)	0.233 ^a (0.085)
<i>C5aR</i>	REST	-0.190 (0.075)	0.085 (0.042)
	DIA	0.068 ^a (0.09)	0.222 (0.075)
	CTX	0.102 ^a (0.100)	0.219 (0.114)
<i>IKKα</i>	REST	-0.014 (0.079)	0.054 (0.042)
	DIA	-0.033 (0.093)	0.044 (0.061)
	CTX	0.016 (0.087)	0.031 (0.054)
<i>IL8</i>	REST	-2.085 (0.214)	-1.432 (0.263)
	DIA	0.21 ^a (0.152)	0.213 ^a (0.116)
	CTX	-0.267 ^a (0.099)	-0.096 ^a (0.153)
<i>MAP3K7</i>	REST	-0.229 (0.052)	-0.071 (0.078)
	DIA	-0.261 (0.070)	-0.340 ^a (0.061)
	CTX	-0.232 (0.083)	-0.146 (0.040)
<i>MD2</i>	REST	-0.104 (0.091)	0.003 (0.046)
	DIA	-0.033 (0.090)	-0.085 (0.031)
	CTX	-0.015 (0.088)	-0.146 (0.045)
<i>MyD88</i>	REST	0.092 (0.088)	0.201 (0.034)
	DIA	0.081 (0.095)	0.128 (0.045)
	CTX	0.178 (0.085)	0.117 (0.039)
<i>PIK3</i>	REST	0.061 (0.096)	0.144 (0.029)
	DIA	-0.198 ^a (0.130)	-0.110 ^a (0.075)
	CTX	-0.130 (0.072)	-0.168 ^a (0.059)
<i>RELA</i>	REST	-0.521 (0.087)	-0.255 (0.094)
	DIA	-0.541 (0.088)	-0.442 (0.057)
	CTX	-0.404 (0.123)	-0.481 (0.104)
<i>Syk</i>	REST	-0.263 (0.067)	-0.257 (0.083)
	DIA	-0.242 (0.096)	-0.630 ^a (0.086)
	CTX	-0.300 (0.100)	-0.436 (0.057)
<i>TICAM-1</i>	REST	-0.789 (0.083)	-0.631 (0.086)
	DIA	-0.612 (0.168)	-0.533 (0.163)
	CTX	-0.621 (0.164)	-0.785 (0.128)
<i>TLR4</i>	REST	-0.422 (0.084)	-0.265 (0.048)
	DIA	0.098 ^a (0.071)	0.175 ^a (0.092)
	CTX	0.010 ^a (0.094)	0.220 ^a (0.057)
<i>TRAF6</i>	REST	-0.316 (0.079)	-0.061 (0.038)
	DIA	-0.401 (0.087)	-0.206 (0.043)
	CTX	-0.383 (0.120)	-0.217 (0.077)
<i>TRIAD3A</i>	REST	-0.020 (0.053)	0.110 (0.025)
	DIA	-0.015 (0.073)	-0.035 (0.084)
	CTX	0.142 (0.089)	0.120 (0.039)

^aSignificant difference in gene expression between REST and DIA or REST and CTX in each lactation stage.

¹REST = unstimulated neutrophils; DIA = diapedesis; CTX = chemotaxis.

Table 4. Fold decrease of differently expressed genes in neutrophils from cows in early lactation¹

Gene	Fold decrease
Unstimulated neutrophils	
<i>ATF3</i>	1.36
<i>C5aR</i>	1.21
<i>IL8</i>	1.57
<i>RELA</i>	1.20
<i>TRAF6</i>	1.19
Diapedesis	
<i>Syk</i>	1.31

¹Only those genes are shown for which the difference between early- and mid-lactating cows was significant ($P < 0.05$). In the fold column, the downregulation of the gene is shown compared with the expression in neutrophils from ML cows. No significant differences in gene expression were found between early- and mid-lactating cows after chemotaxis.

the ML cows were fully competent and these immune-competent cells were used as the normal control group for comparison. Next, the effect of lactation stage and DIA or CTX on the gene expression of selected members from the TLR4 pathway was analyzed.

Effect of Lactation Stage on TLR4 and NF- κ B Signaling in Unstimulated Neutrophils

The mRNA transcription levels in unstimulated neutrophils were compared between cows in EL and ML. In unstimulated neutrophils, no effect for lactation stage was found for the genes of the receptor complex *TLR4/CD14/MD2*, *MyD88*, *TICAM-1*, *MAP3K7*, *IKK α* , and *Syk* (Tables 3 and 4). However, the mRNA transcription of *TRAF6*, *RELA*, and *IL8* was decreased in neutrophils from EL cows. Expression of the endogenous inhibitors of the TLR4 pathway, *TRIAD3A* and *PI3K*, was not influenced by lactation stage, whereas expression of activating transcription factor gene, *ATF3*, was lower in EL cows compared with the ML cows (Tables 3 and 4).

Chemotaxis through a porous membrane did not result in significant different mRNA expressions between cows in EL and ML (Tables 3 and 4). Lactation stage had no effect on the TLR4 pathway in the group of neutrophils that underwent DIA except for *Syk*, which was expressed at a lower level in ML cows (Tables 3 and 4).

Effect of Migration on TLR4 and NF- κ B Signaling in Neutrophils

Within each stage of lactation, the mRNA expression of the selected genes from unstimulated neutrophils was compared with the expression level after DIA or CTX.

In EL cows, significant upregulation of *TLR4*, *ATF3* and *IL8* was observed in neutrophils, after DIA as well as CTX (Tables 3 and 5; $P < 0.05$). The TLR4 inhibitor *PI3K* was significantly downregulated ($P < 0.05$) after DIA, whereas CTX did not influence its expression (Tables 3 and 5). No effect was seen of DIA or CTX on the expression of *CD14*, *MD2*, *MyD88*, *TRAF6*, *TICAM-1*, *TRAF6*, *IKK α* , *MAP3K7*, *RELA*, or *TRIAD3A* (Tables 3 and 5).

In ML cows, DIA and CTX induced a significant upregulation of *TLR4*, *CD14*, *ATF3*, and *IL8* (Tables 3 and 5; $P < 0.05$) and a downregulation of *PI3K* (Table 4; $P < 0.05$). In contrast, mRNA levels of *MAP3K7* and *Syk* were significantly downregulated after DIA (Tables 3 and 5; $P < 0.05$), whereas CTX did not alter the mRNA expression of these genes (Tables 3 and 5). No effect of DIA or CTX was seen on the mRNA expression of *MD2*, *MyD88*, *TICAM-1*, *TRAF6*, *IKK α* , *RELA*, or *TRIAD3A* (Tables 3 and 5).

Effect of Lactation Stage and Migration on C5aR

As the bovine complement factor C5a was used as a chemoattractant for the DIA and CTX assays, the expression of the C5a receptor, *C5aR*, was analyzed in both lactation stages in unstimulated neutrophils and after DIA/CTX.

In unstimulated neutrophils, an effect of lactation stage was observed with significantly lower *C5aR* expression in the immune-compromised neutrophils from EL cows (Figure 2; $P < 0.05$) but not in ML neutrophils (Tables 3 and 4). Diapedesis or CTX increased the mRNA expression of *C5aR* in EL cows ($P < 0.05$) but not in ML cows.

DISCUSSION

To the best of our knowledge, this is the first study evaluating the expression of genes of the TLR4 pathway

Table 5. Fold change of differently expressed genes in neutrophils from early-lactating (EL) and mid-lactating (ML) cows after migration¹

Gene	Chemotaxis		Diapedesis	
	EL	ML	EL	ML
<i>ATF3</i>	0.42↑	0.54↑	0.38↑	0.63↑
<i>CD14</i>	—	0.60↑	—	0.71↑
<i>C5aR</i>	0.82↑	—	0.84↑	—
<i>IL8</i>	0.28↑	0.40↑	0.20↑	0.32↑
<i>MAP3K7</i>	—	—	—	1.20↑
<i>PIK3</i>	—	1.24↓	1.20↓	1.20↓
<i>Syk</i>	—	—	—	1.30↓
<i>TLR4</i>	0.74↑	0.71↑	0.70↑	0.74↑

¹Only those genes are shown for which the difference between resting and migrated neutrophils was significant ($P < 0.05$). ↓ indicates a downregulation and ↑ an upregulation of the gene expression compared with the expression in resting neutrophils before migration.

in functionally characterized neutrophils between dairy cows during EL and ML. Messenger RNA originates from active genes and is used as a template for proteins that determine the neutrophilic phenotype. Although mRNA expression can be used to investigate intracellular events in the neutrophil, it should be mentioned that mRNA undergoes posttranscriptional modifications; thus, measuring mRNA alone has certain limitations when trying to understand innate immunity. Still, it was our specific goal to initiate a study to investigate the expression of genes of the TLR4 pathway in immune-compromised neutrophils, based on the hypothesis that this pathway could be involved in postpartum *E. coli* mastitis (De Schepper et al., 2008). Recent studies suggest an important role of *TLR4* in endometritis and mastitis, which are common postpartum diseases in dairy cow. Herath and coworkers (2009) observed an upregulation of *TLR4* in the endometrium of infertile animals with persistent endometritis compared with fertile, healthy postpartum cows. They hypothesize that an unlimited innate response could be at the basis of endometritis development. A similar result was found by Petzl et al. (2008) in mammary gland tissue from infected udder quarters in an *E. coli*-induced mastitis model. Interestingly, a relationship between impaired functions of peripheral blood neutrophils during the periparturient period and metritis (Hammon et al., 2006) on the one hand and mastitis (Heyneman et al., 1990) on the other hand has been found. Nothing is known yet about the expression of the TLR4 pathway in immune-compromised neutrophils. Therefore, this preliminary study was set up to compare the expression of TLR4-related genes in immune-compromised and immune-competent neutrophils. First, in vitro leukocyte function tests were performed to characterize the neutrophils. In agreement with previous reports (Burvenich et al., 2003), we observed that blood neutrophils isolated from cows in EL showed a decreased CTX, DIA, and phagocytosis compared with neutrophils isolated from cows in ML. Oxidative burst, measured by ROS production after PMA stimulation, had a tendency to be lower in neutrophils from EL cows but this could not be statistically confirmed. Failure to identify this statistical difference might be due to the individual variation of the cows, the relatively small sample size, or the method. The generation of ROS is mediated by a membrane-associated complex referred to as NADPH oxidase (Babior, 1999). This enzyme complex can be activated by zymosan and protein kinase C activators such as PMA. Its activation leads to the production of intracellular and extracellular ROS. Rinaldi et al. (2007) analyzed different assays for the measurement of ROS in bovine neutrophils. Those authors concluded that luminol-dependent chemiluminescence reflected in-

tracellular increases in ROS production or extracellular increases in ROS other than superoxide and hydrogen peroxide. This might explain why we could not detect a significant decrease in respiratory burst in EL cows.

Based on the functional profiles, neutrophils from EL cows were considered immune-compromised and those from ML cows were considered immune-competent and were used as a control group. Next, we wanted to know if this immune-compromised neutrophil status was accompanied by alterations in the mRNA expression of TLR4-related genes.

In unstimulated neutrophils, mRNA expression of TLR4-associated genes was virtually indistinguishable between EL and ML cows, with the exception of a decreased expression of *TRAF6*, *RELA*, *ATF3*, and *IL8* in neutrophils from EL cows. The *TRAF6* gene acts downstream in the TLR4 pathway and is a key gene in the activation of *NF- κ B*, which eventually leads to the production of inflammatory cytokines (Inoue et al., 2007). We also observed a significant decrease in the transcription of the cytokine *IL8*, tempting us to hypothesize that the decreased *TRAF6* expression was translated in a decreased *IL8* expression. In a microarray study comparing the expression levels of several genes in blood neutrophils at parturition and 7 d before parturition, a similar downregulation of *TRAF6* expression was reported but was not accompanied by decreased *IL8* expression (Madsen et al., 2004). This discrepancy could be due to the fact that in the present study, neutrophils were used from cows 3 d postparturition and 149 to 151 d postparturition. It could also be explained by the fact that not all genes expressed by neutrophils were analyzed in our study. For example, it has been shown that TLR4 signaling is controlled by intracellular regulators such as *PI3K*, *ATF3*, and *TRIAD3A*, but membrane-associated receptors such as *ST2* (a toll-IL-1R domain receptor) and *TRAILR* (a tumor necrosis factor receptor) have also been described to regulate TLR4 signaling (Liew et al., 2005). This suggests that complex regulatory networks exist and that the downregulation of *IL8* could be caused by regulatory genes not included in the present study.

Next, we wanted to know if migration, consisting of DIA and CTX, affects *TLR4* signaling in neutrophils. The mammary epithelial model system used in this study has been successfully used in our laboratory to study neutrophil DIA in vitro. It consists of a monolayer of BME-UV cells seeded on a collagen-coated membrane insert and uses C5a as a chemoattractant (Smits et al., 1996). For CTX, a similar model was used without the epithelial monolayer and collagen coating. Because C5a was used for the migration experiments, one could argue that the chemoattractant by itself was responsible for the observed changes in mRNA expres-

sions. However, in a previous study at our laboratory, the direct effect of C5a on the gene expression of the TLR4 pathway in neutrophils was determined and it was found that gene expressions were not influenced by the lactation stage [M. G. H. Stevens, L. J. Peelman, B. De Spiegeleer, A. Pezeshki, G. R. Van De Walle, L. Duchateau, C. Burvenich, and R. Rainard (Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, Nouzilly, France), unpublished data]. Therefore, we conclude that the observed differences in gene expression after migration seen in the present study between neutrophils from EL and ML cows are not caused by the chemoattractant but are the result of neutrophil migration.

The salient findings were that both DIA and CTX induced the mRNA transcription of *TLR4*, *IL8*, and *ATF3* in neutrophils from EL and ML cows. The primary function of the TLR4 receptor is to recognize PAMP from invading pathogens (Beutler et al., 2001). Downstream signaling events will then ultimately result in the production of proinflammatory cytokines such as IL-8. To our knowledge, nothing is known yet about the effect of DIA or CTX on *TLR4* in neutrophils, but our data suggest that neutrophil migration is able to regulate the expression of the TLR4 receptor to prepare the neutrophils for the recognition of pathogens and triggers the transcription of *IL8*.

TLR4's co-receptor *CD14* was significantly upregulated in immune-competent neutrophils after DIA and CTX, but no upregulation was seen in the immune-compromised neutrophils. No explanation for this unexpected observation of a *TLR4* upregulation in the absence of *CD14* upregulation in immune-compromised neutrophils can be given, but it might suggest that these neutrophils have dysfunctional LPS sensing after DIA, which has been hypothesized by De Schepper et al. (2008). However, it should be taken into account that upregulation at the mRNA level does not necessarily translate into a functional effect and therefore these observations should be confirmed at protein level.

The TLR4 pathway is strictly regulated, and intracellular and transmembrane proteins have been identified that negatively regulate TLR4 signaling (Liew et al., 2005). For example, *ATF3* functions as a negative regulator of the TLR4 receptor by inhibiting NF- κ B (Gilchrist et al., 2006). Here, we observed an increased level of *ATF3* mRNA in neutrophils after DIA, which may suggest a protective feedback on the NF- κ B signaling pathway. A tendency for decreased expression of *RELA* was noted but further research is needed to elucidate this.

Although migration induced different changes in mRNA expression in neutrophils from EL and ML cows, mRNA expression after migration of most of the

TLR4-related genes was not significantly different except for that of *Syk*. We found that DIA but not CTX induced significant downregulation of *Syk* in immune-competent neutrophils, whereas DIA did not change *Syk* expression in the immune-compromised neutrophils. No explanation has been found to date for the unchanged *Syk* expression after DIA in immune-compromised neutrophils, which was in contrast to findings in the immune-competent neutrophils. However, *Syk* could be an attractive candidate for further research because it has been reported that type I hypersensitivity reactions such as allergic rhinitis and systemic anaphylaxis seem to partially depend on *Syk* activation, and targeting *Syk* has therefore been proposed as a means for treatment (Wong et al., 2004).

CONCLUSIONS

We performed a genomic scan by qPCR analysis of the TLR4 pathway in neutrophils from EL and ML cows. To our best knowledge this is the first study to show that neutrophil migration (DIA and CTX) changes mRNA expression in bovine neutrophils. Moreover, migration differently influenced the mRNA expression of *TLR4*-related genes in EL and ML cows. This study shows that the perturbations in neutrophil functions during EL are accompanied by modulation of TLR4-related genes. These data can contribute to the understanding of the mechanisms to explain the relationship between stage of lactation and risk of severe *E. coli* mastitis.

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