Distinct neutrophil C5a receptor inflammatory events in cows initiated by chemoattractant C5a and lipopolysaccharide around parturition and in mid lactation

X. Boulougouris,¹ C. Rogiers,¹ M. Van Poucke,¹ B. De Spiegeleer,² L. J. Peelman,¹ L. Duchateau,¹ and C. Burvenich^{1*}

¹Animal Genetics Laboratory, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, Merelbeke, Belgium

²Drug Quality and Registration (DruQuaR) Group, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, Ghent, Belgium

ABSTRACT

In neutrophils, toll-like receptor and complement component 5a (C5a) signaling are critical pathways regulating innate immunity. In cows, not much is known about the second C5a receptor, complement component 5a receptor 2 (C5AR2). It is an interesting player in sepsis treatment because it is considered to have an anti-inflammatory effect during normal inflammation. Periparturient cows are prone to severe infections, and the objectives of this study were to investigate the expression and functionality of C5AR2 during peripartum. We investigated the effect of 2 major inflammatory stimuli, C5a and lipopolysaccharide (LPS), on the expression of a selected number of genes (*C5AR1*, *C5AR2*, *TLR4*, *ITGAM*, *COX2*, and *CXCL8*) and functions linked to these receptors. Overall, *TLR4*, *ITGAM*, and *C5AR2*, all of which are involved in early inflammation, showed a lower expression in periparturient cows. However, an overall lower expression seems not to be the only explanation for the increased risk of sepsis in periparturient cows. Normally, in response to inflammation and as seen in the mid-lactation group, the expression of these genes increases after stimulation with LPS. However, in periparturient cows, stimulation with LPS led to a decrease in expression of these receptors, indicating a different response of neutrophils in response to LPS during this period. A decrease in *ITGAM* (coding for CD11b) expression complicates correct neutrophil localization and phagocytosis. Its downregulation upon stimulation might be detrimental for adequate eradication of the pathogen and might increase the risk of an imbalanced inflammation; *C5AR2* seems to play a central role in this altered response. In

addition, myeloperoxidase (MPO) activity in periparturient cows is lower in response to C5a stimulation. It has been suggested that MPO plays an important role in neutrophil shutdown and, thereby, timely resolution of inflammation. A decreased MPO activity might thus prolong the inflammatory reaction of the neutrophils. This finding was supported by the increased viability of the neutrophils obtained from periparturient cows. Even after stimulation, we found a lower caspase-3 activity in this group, indicating that they might be activated for a longer time compared with the neutrophils from mid-lactation cows. Accordingly, these alterations might contribute to a temporal mismatch in inflammatory responses, as often seen in severe periparturient infections.

Key words: peripartum, neutrophil, C5AR2, CD11b

INTRODUCTION

During the periparturient period, cows are prone to developing severe mastitis in response to intramammary *Escherichia coli* infections. It is now generally accepted that an altered function of blood neutrophils is involved in the underlying mechanisms of the temporary weakening of the cow's innate defense around parturition. Decreased killing by production of reactive oxygen species and reduced migration to the site of infection have especially been observed during this period (Burvenich et al., 2003).

Complement component C5a (C5a) is a critical early mediator in the development of severe *E. coli* mastitis (Shuster et al., 1997). Its effect on neutrophils can be mediated by 2 receptors, C5aR and C5AR2 (Guo and Ward, 2005). In contrast to the well-described, global proinflammatory role of C5aR, the role of the second C5a receptor, C5AR2, is still under debate. In non-sepsis models, the biological role of C5AR2 appears to be limiting the proinflammatory function of C5aR (Li et

Received July 10, 2018.

Accepted October 20, 2018.

*Corresponding author: christian.burvenich@ugent.be

al., 2013). The mechanisms that regulate this delicate balance are an active focus of investigation in human studies. In the mammary gland, however, the exact role of C5a during *E. coli* mastitis is still unclear; C5a is a potent chemoattractant for neutrophils and it promotes survival, thereby contributing to an appropriate localization and protracting neutrophil function in the inflammatory process (Perianayagam et al., 2002). A fast recruitment of neutrophils is necessary to protect the mammary gland efficiently against invading pathogens (Paape et al., 2003), whereas delayed recruitment is correlated with severity of *E. coli* mastitis (Vandeputte-Van Messom et al., 1993).

Moreover, in vitro stimulation of neutrophils with LPS increases neutrophil surface expression of the adhesion molecule CD11b, important for triggering diapedesis and phagocytosis (Chen et al., 2007). In addition, a crosstalk between the C5aR and toll-like receptor (TLR)-4 has been suggested (Stevens et al., 2011b; Holst et al., 2012). It was demonstrated in mice that TLR-4 activation with LPS induces a hypersensitivity to C5a. This hyperresponsiveness was not attributed to an upregulation of C5aR but rather to an inhibition of the C5AR2 receptor (Raby et al., 2011). By downregulating *C5AR2* and its anti-inflammatory function, *C5AR1* proinflammatory effector functions are enhanced, potentially increasing eradication of the pathogen.

Considering the proposed anti-inflammatory role of C5AR2 and the regulatory function of LPS on its expression, these insights might offer a possible explanation for the excessive inflammatory reaction seen in peripartum episodes of *E. coli* mastitis. To our knowledge, the expression pattern of *C5AR2* during the periparturient period is not known. To further elucidate the role of C5a in peripartum mastitis, we hypothesized that *C5AR2* expression is decreased during the periparturient period, leading to a diminished anti-inflammatory effect on C5aR. Therefore, we compared the constitutive expression of the C5a receptors in periparturient and mid-lactation cows. We also hypothesized that a decreased *C5AR2* expression during the periparturient period results in different downstream effects after stimulation with LPS, comprising altered migration and an unbalanced production of inflammatory cytokines and prostaglandins as often observed during periparturient inflammation (Burvenich et al., 2003). We used mid-lactation cows as the control group and investigated the effect of high but physiological doses of C5a and LPS to compare the periparturient expression of 6 key genes in the early stage of neutrophil sensing (*C5AR1*, *C5AR2*, *ITGAM*, *TLR4*) and local inflammation (*COX2*, *CXCL8*). Three major effector functions, potentially linked to the C5AR2 receptor, were analyzed

to further unravel their role in neutrophils during the peripartum period: IL-8 production, myeloperoxidase (MPO) enzyme (EC 1.11.2.2) activity, and apoptosis (Mollnes et al., 2002; Perianayagam et al., 2002; Revello and Waldron, 2012).

MATERIALS AND METHODS

Experimental Protocol

Blood samples from 12 Holstein-Friesian cows were collected at 2 time points in this longitudinal study: (1) within 24 h of calf delivery and (2) during mid lactation ($d\ 135 \pm 7d$). The function of blood neutrophils collected from healthy mid-lactation cows is considered immune competent (Burvenich et al., 2003). Therefore, we considered the results of the analyses performed during mid lactation as controls to interpret the results obtained at parturition. The experimental protocol is schematically presented in Figure 1. This study consisted of 2 parts. The first part consisted of characterization of the obtained neutrophils after isolation. Six cows were sampled after parturition and during mid lactation and characterized by means of their viability after isolation, purity, and maturation state. The second part consisted of analysis of the different response of neutrophils obtained from periparturient and mid-lactation cows upon stimulation with C5a and LPS. Six cows were used for incubation of their neutrophils with different stimuli (C5a, LPS, or both). Unstimulated cells were used as the control. The results of stimulated cells might give us more insight into why cows are more prone to develop a severe inflammatory response specifically upon C5a and LPS exposure. After stimulation, supernatant was collected to evaluate its IL-8 content. The cells were used to analyze gene expression, MPO activity, and caspase-3 activity. Plasma of the cows was used to evaluate the cortisol concentration.

Animals and Blood Sampling

Blood was sampled from the coccygeal vein of clinically healthy Holstein-Friesian cows and collected in EDTA-coated blood collection tubes (Vacutainer, Becton Dickinson, Erembodegem, Belgium). Blood was always sampled between 0700 and 0900 h to avoid circadian influences on neutrophil functionality (Adrover et al., 2016), and the calf was already removed when samples were taken. Sampling was done from January until March (part A) and October until December (part B). The average time of blood sampling after parturition was 12 h. After collection, tubes were inverted 3 times, stored on ice, and processed within 0.5 h of collection. The nominal EDTA concentration was 1.8

mg of EDTA/mL of blood. The animals were housed at the dairy farm of Ghent University (Biocenter AgriVet, Melle, Belgium). All cows had calved normally and showed no clinical signs of periparturient diseases. The animals were kept in freestall housing, were fed TMR, and had access to water ad libitum. Cows were milked with a milk robot (DeLaval VMS, Gent, Belgium) at an average voluntary milking frequency of 2.7 times/d. All cows were free from IMI, and milk SCC from each quarter during mid lactation was less than 200,000 cells/mL. The study was approved by the Ethical Committee before beginning (number EC2012/020). Only multiparous cows were incorporated in the study based on previous study results that indicated an in-

creased prevalence of severe clinical mastitis in multiparous versus primiparous cows (Gilbert et al., 1993; Vangroenweghe et al., 2004a, b). Parity in the current study population ranged from 2 to 6.

Neutrophil Isolation, Characterization, and Viability

Isolation of neutrophils was performed as described by Siemsen et al. (2007). In short, plasma was removed after centrifugation and red blood cells were lysed by adding 50 mL of endotoxin-free purified water. Five milliliters of NaCl (10%; Sigma-Aldrich, Bornem, Belgium) was added and samples were centrifuged at $740 \times g$ for 10 min at room temperature with low braking

Figure 1. Study design. Blood was drawn from the coccygeal vein of 12 healthy dairy cows at the day of parturition (within 24 h after parturition) and 135 ± 7 d postpartum. The study consists of 2 parts. (A) Characterization of the obtained neutrophils by means of determination of their viability, purity of the population, and their maturation state. (B) Stimulation of neutrophils with C5a, LPS, or a combination of both (C5a + LPS). Unstimulated cells (incubated in Hanks' balanced salt solution) were used as a control. The supernatant was checked for differences in IL-8 protein between both groups. The cell lysate was analyzed for gene expression and myeloperoxidase (MPO) activity in the same fashion. A caspase-3 assay was applied to assess neutrophil apoptosis. Finally, plasma was checked for cortisol concentration.

speed. The resulting leukocyte suspension was layered carefully on top of a Histopaque 1077/1119 gradient (Sigma-Aldrich). The final pellet was resuspended in 1 mL of PBS (Sigma-Aldrich). Cell viability was determined in triplicate (3×100 cells were counted) by the Trypan blue (Invitrogen, Merelbeke, Belgium) exclusion test using a Bürker chamber. Neutrophil purity was analyzed by bright-field microscopy with a $100\times$ objective and immersion oil (Axioskop 2 MOT, Carl Zeiss N.V., Oberkochen, Germany) by staining cells with Sangodiff G (Sigma-Aldrich). Purity was also determined in triplicate (3×100 cells were counted).

Stimulation of Neutrophils with C5a and LPS

Neutrophils (2.5×10^7 cells) were challenged with (1) 50 ng/mL of C5a (courtesy of P. Rainard, Institut National de la Recherche Agronomique, Nouzilly, France), (2) 10 µg/mL LPS (from *E. coli* O111:B4, TLR-4 ligand tested, <1% protein and <1% RNA; Sigma-Aldrich) diluted in sterile-filtered PBS (Invitrogen) with 0.1% BSA (Roche Applied Science, Vilvoorde, Belgium), or (3) both. Concentrations of 50 ng/mL C5a are analogous to concentrations elicited during experimental *E. coli* mastitis (Smits et al., 1996). The LPS dose used in this study (10 µg/mL) was within range of doses often used during experimental LPS challenges (Revelo and Waldron, 2012). Unstimulated cells, incubated in Hanks' balanced salt solution (Invitrogen, Merelbeke, Belgium), were used as the control (Figure 1). Before challenge, all reagents were filtered using Acrodisc units (0.2-µm membrane; Pall Life Sciences, Saint-Germain-en-Laye, France). Cells were incubated in a humidified incubator (5% CO₂, 37°C) for 2 h. All efforts were made to reduce endotoxin contamination by use of sterile and endotoxin-free materials. After incubation, samples were centrifuged at $2,500 \times g$ for 10 min at room temperature. Supernatant was collected and stored immediately (−80°C) until analysis. The cellular part was resuspended in PBS and aliquoted for RNA isolation and protein extraction.

Gene Expression

RNA Extraction and cDNA Synthesis. Total RNA was isolated immediately after incubation, and the same kit was used throughout the experiment. Isolation was done using Total RNA Isolation Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The RNA was dissolved in 15 µL of preheated MilliQ water (65°C; VWR International, Leuven, Belgium). Genomic DNA was removed by adding 4 µL of RQ1 RNase-free DNase (0.5 U/µL; Promega, Leiden, the Netherlands) and 2 µL of RQ1 DNase 10× Reaction Buffer (Pro-

mega) to 14 µL of RNA (30 min, 37°C). An RQ1 stop solution (2 µL) was added to end the reaction (10 min by 65°C), and the RNA was purified by spin-column purification (Amicon Ultra 0.5-mL 100 K Centrifugal Filter; Merck, Millipore, MA). The RNA concentration of the samples (2.5×10^6 neutrophils) was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, Sint-Pieters-Leeuw, Belgium) and ranged from 3.96 to 9.63 ng/µL. The RNA quality was assessed by Experion analysis using High Sense chips (Bio-Rad, Nazareth-Eke, Belgium). The RQI (RNA quality indicator) values of RNA from neutrophils ranged from 3.7 to 9.3.

Absence of DNA in the samples was confirmed by minus reverse transcription PCR control. This was performed on the Eppendorf Mastercycler PCR system (Eppendorf, Rotselaar, Belgium). A PCR mix was made in a total volume of 10 µL/sample, containing 1 µL of sample, 0.1 µL of Taq DNA Polymerase (5 U/µL; Roche Applied Science), 0.2 µL of dNTP Mix (10 mM each; Bioline, London, UK), 1 µL of primer (HPRT1 ± 1, 5 µM each; Integrated DNA Technologies, Leuven, Belgium), 1 µL of 10× FastStart Taq DNA Polymerase Buffer (Roche Applied Science), and 6.7 µL of ultrapure water (Milli-Q, Merck Millipore, Brussels, Belgium). The PCR program consisted of an initiation step of 4 min at 95°C followed by 40 amplification cycles (denaturation for 15 s at 95°C, annealing for 15 s at 60°C, and extension for 30 s at 72°C) and a final 2-min elongation step at 72°C. The amplification products were additionally analyzed on a 2% ethidium bromide-stained agarose gel (150 V, 20 min, results not shown), and DNase treatment was repeated if DNA was still present.

A total of 10 µL of DNase-treated total RNA was used for generating cDNA using random hexamers and oligo dT primers (each 0.4 µg/reaction; IDT, Leuven, Belgium) by means of the Improm-II Reverse Transcription System (Promega). The primer-template mix was thermally denatured by incubation at 70°C (5 min) followed by 5 min of incubation on ice. Then, 4 µL of Improm-II 5× reaction buffer (Promega), 2.4 µL of MgCl₂ (25 mM), 1 µL of dNTP Mix (10 mM each; Bioline), and 1 µL of Improm-II reverse transcriptase (20 U/µL; Promega) were added. The final volume of 20 µL was incubated for 5 min at 25°C (primer annealing), 60 min at 42°C (first-strand synthesis reaction), and 15 min at 70°C (inactivation of reverse transcriptase). Samples were diluted 5 times and stored at −20°C.

Primer Design. The primers for the target and reference genes were based on the mRNA sequences of the bovine genes (<http://www.ncbi.nlm.nih.gov>); the primers were designed using Primer 3 software (http://www.sk.embnnet.org/cgi-bin/primer3_www.cgi). The

Table 1. Primers of target and housekeeping genes

Gene	NCBI gene ID	NCBI accession no.	Sequence ¹	Amplicon length (bp)	PCR efficiency (%)	Annealing temperature (°C)
Housekeeping genes						
<i>ACTB</i>	280979	NM_173979.3	F: GCGGCATTACAGAACTAC R: TCTGCTGGAAGGTGGACAG	249	94.2	60
<i>H2A</i>	506900	NM_001205596.1	F: GTCGTGGCAAGCAAGGAG R: GATCTCGGCCGTTAGGTACTC	182	89.4	60
<i>HPRT1</i>	281229	NM_001034035.2	F: TGCTGAGGATTTGGAGAAGG R: CAACAGGTTCGGCAAAGAACT	154	98.2	60
<i>YWHAZ</i>	287022	BM446307.1	F: GCATCCACAGACTATTTCC R: GCAAAGACAATGACAGACCA	120	85.4	58
Target genes						
<i>TLR4</i>	281536	NM_174198.6	F: CAGGGCAGGGAAAGTCAA R: AGGAAAAGTGAGCCAAGACC	203	98	58
<i>C5AR1</i>	493645	NM_001007810.3	F: ACTGGTCTTTGGTGATGCT R: GTGGGAAGTGGTCTATGTGG	249	89.3	60
<i>COX2</i>	3283880	AF031698.1	F: TGACCAGAGCAGAGAGATGAAA R: CAGAAGGGCGGGATAAAAC	169	98.9	56
<i>CXCL8</i>	280828	NM_173925.2	F: CTGGCTGTGTGCTCTCTTGG R: CACTTTTCCTTGGGGTTTAGG	239	94.3	54
<i>C5AR2</i>	522144	NM_001077947.1	F: GGGCCTGGAGTTTCAGAGT R: GACATGCAGGAGCCATCAG	183	88.9	60
<i>ITGAM</i>	407124	NM_001039957.1	F: TCTACTCCAGCAACCCAGGA R: AACCTCTTCATCCGCTCAAA	129	91.7	58

¹F: forward primer; R: reverse primer.

optimal annealing temperature was set at 60°C, the optimal GC content was set at 50%, and the maximal self-complementarity was kept as low as possible. The presence of secondary structures in the amplicon was checked using Mfold¹ (<http://www.bioinfo/rpi.edu/applications/mfold/dna>), and the specificity was checked using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST>). Primers were ordered from IDT. Table 1 lists the target genes, primer conditions, and amplicon information.

Quantitative Real-Time PCR Analysis. Quantitative real-time PCR was performed using SYBR Green chemistry (SYBR Green PCR Master Mix; Applied Biosystems, Ghent, Belgium). The increase in fluorescence of the SYBR Green dye was monitored using an iCycler (Applied Biosystems). A PCR mix was made containing 1 µL of each primer solution (forward and reverse, each 5 µM), 5 µL of 2× SYBR Green I Master Mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland), 2 µL of cDNA sample, and 2 µL of ultrapure water (Milli-Q, Merck Millipore). The PCR program consisted of an initiation step (3 min, 95°C) followed by 40 cycles of amplification (denaturation at 95°C for 30 s, annealing and elongation for 40 s at the optimal annealing temperature, and detection of fluorescent signals generated by SYBR Green I–double-stranded DNA binding). During each run, a 5× dilution series was included to create a relative standard curve, 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. Optimal annealing temperatures were determined experimentally by gradient quantitative PCR. The PCR efficiencies were between 85.40 and 98.95%, 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 and target genes on all samples. Six candidate reference genes were selected based on previous research (Goossens et al., 2005; Stevens et al., 2011a), as encouraged by the MIQE guidelines (Bustin et al., 2009). Obtained relative quantities, calculated based on the delta-delta Ct method taking into account quantitative PCR efficiencies (Pfaffl, 2001), were normalized using the geometric mean of the 4 most stable reference genes (*HPRT1*, *H2A*, *YWHAZ*, and *ACTB*; Table 1; Vandesompele et al., 2002). For all statistical analyses, these normalized expression data were converted to logarithmic (base 10) values to make the data distribution symmetrical and to obtain more accurate and relevant results (Helleman and Vandesompele, 2011). The pairwise variation (V3/4) of these genes using geNorm (<https://genorm.cmgg.be>) was 0.168; hence, they were deemed suitable as an internal control (Vandesompele et al., 2002). Data are presented as means ± standard error of the mean.

Functional Assays

Protein Extraction and Protein Concentration.

Before the MPO and caspase-3 assay, 2.5×10^6 neutrophils were lysed using Mammalian Protein Extraction Reagent (M-PER; ThermoFisher Scientific, Asse, Belgium) to determine the protein concentration. The M-PER uses a nondenaturing detergent that is compatible with enzyme assays. Protease inhibitor cocktail (Sigma-Aldrich) was added to the cells before lysis, and the mixture was shaken gently for 10 min. Debris was removed by centrifugation at $14,000 \times g$ for 15 min at 4°C , and supernatant containing the protein fraction was stored immediately at -80°C until analysis. The protein concentration of the cytosol extract was determined by the Bradford assay using Coomassie Brilliant Blue (ThermoFisher Scientific) according to manufacturer's protocol. The average protein concentration for 2.5×10^6 neutrophils diluted in M-PER was 585 and 1,398 $\mu\text{g/mL}$ during the periparturient period and mid lactation, respectively. Absorbance was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science).

MPO Assay. Detection of chlorination activity of MPO was performed using the EnzChek Myeloperoxidase Activity Assay Kit (ThermoFisher Scientific) according to the manufacturer's protocol. A total of 50 μL of M-PER-lysed sample (an equivalent of 1.25×10^5 neutrophils that contained different protein concentration; see above) was incubated for 30 min at room temperature, protected from light. The fluorescence intensity of each sample was measured using excitation at 492 nm and emission at 535 nm (TriStar² LB 942 Multidetector Microplate Reader; Berthold Technologies, Vilvoorde, Belgium). The fluorescence intensity of the negative control standard was subtracted from all of the experimental samples.

Caspase-3 Activity. A caspase-3 (EC 3.4.22.56) assay was used to estimate the viability of our neutrophils after stimulation. Activation of caspase-3 and related caspases leads to the morphological and biochemical changes that drive the apoptotic process (Nicholson, 1999). Caspase activity was determined by measuring the proteolytic DEVDase activity of caspase-3 and related caspases using the Caspase-3 assay kit (Abcam). Samples were thawed on ice and diluted according to manufacturer's protocol to obtain 50 μg of protein/50 μL of cell lysis buffer. Based on the protein concentration, an appropriated number of cells was used to obtain 50 μg of protein for each sample (see above). The cells were lysed before the procedure. (For detailed description of lysis, assay procedure, and data analysis, see the manufacturer's protocol.) After incubation, ab-

sorbance was read at 405 nm using a 96-well Anthos 2010 microplate reader (Biochrom, Cambridge, UK).

IL-8 ELISA. After stimulation of the cells, levels of IL-8 in the supernatant were analyzed with an ELISA kit for bovine IL-8 (Gentaur, Brussels, Belgium) according to the manufacturer's protocol. The manufacturer declared no significant cross-reactivity or interference between bovine IL-8 and analogs. Samples and standards were added to the appropriate microtiter plate wells coated with biotin-conjugated IL-8 antibodies and incubated for 1 h at 37°C . Next, avidin-conjugated horseradish peroxidase was added to the wells and the samples were incubated at 37°C . Twenty minutes after addition of tetramethylbenzidine substrate, the color reaction was stopped by adding a stop solution. The color change was measured spectrophotometrically at 450 nm. The concentration of IL-8 was determined by comparing the optic density of the samples with the standard curve. The intra-assay coefficient of variability was 6.1% (an intra-assay coefficient of variability of less than 10 is considered acceptable).

Plasma Cortisol Levels. To determine cortisol concentration in plasma, 50 μL of each sample was analyzed by ELISA according to the manufacturer's protocol (Cortisol Competitive ELISA Kit; ThermoFisher Scientific, Asse, Belgium). Cortisol conjugate and antibody were added to the samples, and the plate was allowed to incubate at room temperature for 1 h. The plate was washed and tetramethylbenzidine substrate was added to each well. After 30 min of incubation, the plate was read at 450 nm using a 96-well Anthos 2010 Microplate Reader (Biochrom). All samples and standards were analyzed in duplicate, and absorbance of tetramethylbenzidine was accounted for substrate background and subtracted from the absorbance values of the wells being assayed. A standard curve was used to determine the concentration of the samples. The intra-assay coefficient of variability was 3.52% (an intra-assay coefficient of variability of less than 10 is considered to be acceptable).

Statistical Analysis

A mixed model with cow as random effect and stimulus, lactation stage, and interactions as fixed effects was fitted to the target gene expression data and the MPO activity, caspase-3 enzymatic activity, and IL-8 production by the neutrophils. Within the same model, the effect of the lactation stage at each level of stimulation was studied. Hypothesis testing was done at the 5% significance level. Pearson correlation coefficients between gene expression levels and functional tests were calculated at 5 and 1% significance levels.

RESULTS

Neutrophil Isolation, Characterization, and Viability

As determined by trypan blue, neutrophil viability after isolation was >99% for both periparturient and mid-lactation cows. Before the study, neutrophils were tested for their viability after 2-h incubation with the same condition as used in this study. Viability remained high after 2 h: 93.37 and 96.12% of neutrophils obtained from periparturient and mid-lactation cows, respectively, were considered viable by the trypan blue exclusion test.

Purity of the neutrophils was 93 and 99% in periparturient and mid-lactation samples, respectively. In the neutrophil population, 74% were mature segmented neutrophils in periparturient cows and 88% were mature segmented neutrophils in mid-lactation cows.

Gene Expression

As we were interested in the differences between the lactation stages, we focus on these results rather than the effect of stimulation compared with nonstimulated cells. Our mid-lactation cows responded as we expected and as described in the literature (Paape et al., 1996, 2003; Revelo and Waldron, 2012). Therefore, they were used as the control group with which to compare the results of the periparturient cows.

Main Effects. We found a strong effect of lactation stage for several genes regardless of the stimuli applied. These results are discussed below. We also checked for the interaction between lactation stage and stimulation, but no significant interaction was found. This means that, for our study population, periparturient and mid-lactation cows globally reacted similarly upon stimulation. The results of the gene expression patterns are depicted in Figure 2.

Effect of Lactation Stage. The expression of *TLR4* was significantly affected by lactation stage ($P = 0.047$; Figure 2A, left column), with an overall lower expression of *TLR4* in periparturient cows. When comparing each stimulus between both lactation stages separately, we found that *TLR4* expression was lower in the periparturient cows in any of the applied stimuli, but we did not find significant differences (Figure 2A, right column).

The overall *C5AR2* expression (Figure 2E, left column) was also significantly lower in the periparturient cows ($P = 0.037$) compared with mid-lactation cows. When comparing the separate effects of the stimuli (Figure 2E, right column), only stimulation with LPS (without C5a) resulted in a significantly different expression between the 2 lactation stages, with a lower

expression ($P = 0.041$) of *C5AR2* in periparturient cows. Lipopolysaccharide induced a reduction of *C5AR2* expression in both lactation stages, but the effect was more pronounced in periparturient cows, which resulted in a significantly different expression.

This was also the case for overall *ITGAM* expression (Figure 2C, left column), with a lower expression of *ITGAM* during the periparturient period ($P < 0.001$). Here, significantly lower *ITGAM* expression was found in all stimulus groups in periparturient cows (nonstimulated: $P = 0.0255$; C5a: $P = 0.0158$; LPS: $P = 0.0003$; C5a + LPS: $P = 0.006$; Figure 2C, right column). No significant effect of lactation stage was observed for *COX2* ($P = 0.127$), *CXCL8* ($P = 0.309$), and *C5AR1* ($P = 0.204$; Figure 2B, D, F).

Correlation Coefficient Between the Gene Expression Levels and the Functional Tests. Correlations were calculated between the gene expression levels (Table 2). We focused on the expression of *C5AR1* and *C5AR2* and found that the expression of both of these C5a receptors is highly correlated ($P < 0.0001$). Concerning the *C5AR1* receptor, we also found a high correlation with *TLR4* ($P < 0.0001$) and *CXCL8* ($P < 0.0001$). The expression of *C5AR2*, on its turn, also showed a high correlation with *CXCL8* ($P < 0.0001$). No significant correlation was found between gene expression of the C5a receptors and the functional tests (results not shown).

C5AR1/C5AR2 Ratio. We also investigated the expression of C5aR relative to the expression of *C5AR2* (Figure 3). This was done by analyzing the *C5AR1/C5AR2* mRNA expression ratio of the periparturient cows and comparing it with the expression ratio in mid-lactation cows. Remarkably, although both *C5AR1* (albeit not significantly so) and *C5AR2* ($P = 0.037$) are downregulated in periparturient cows (see above), we found a lactation stage effect for this expression ratio ($P = 0.0306$). This indicates that despite the lower expression of both receptors during the periparturient period, *C5AR1* is relatively more expressed compared with *C5AR2* (Figure 3, left column). When comparing each stimulus between both lactation stages separately, we did not find significant differences for our study population. However, *C5AR1/C5AR2* mRNA expression ratio was higher in the periparturient cows in any of the applied stimuli, with the biggest difference after LPS stimulation (Figure 3, right column).

Functional Assays

Similar to gene expression profiles, the discussion of the results of the enzyme assays is limited to the differences between the lactation stages.

Figure 2. Quantitative real-time PCR analysis of the expression of (A) *TLR4*, (B) *C5AR1*, (C) *ITGAM*, (D) *COX2*, (E) *C5AR2*, and (F) *IL8* in neutrophils obtained from periparturient (within 24 h after calving) and mid-lactation (135 ± 7 DIM) cows after 2 h of stimulation with various stimuli. Bars represent mean expression (logarithmic scale) $\pm$ SEM normalized by the expression of 4 reference genes (*HPRT1*, *H2A*, *YWHAZ*, and *ACTB*) as described by Vandesompele et al. (2002). The first column denotes the effect of lactation stage, and the second column denotes the effect of lactation stage per stimulus. As we were interested in the differences between the lactation stages, the effect of stimulation compared with nonstimulated cells is not discussed in this figure. Nonstimulated cells were used as a control. Control: no stimulus (Hanks' balanced salt solution); C5a: 50 ng/mL; LPS: 10 μ g/mL; LPS+C5a: C5a (50 ng/mL) + LPS (10 μ g/mL). Light gray bars represent neutrophils obtained from periparturient cows, and dark gray bars represent neutrophils obtained from mid-lactation cows. Significant differences between periparturient and mid-lactation cows are denoted with stars (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 2. Correlation coefficients between the gene expression levels.

Gene	r^1	
	<i>C5AR1</i>	<i>C5AR2</i>
<i>C5AR1</i>	—	0.7014**
<i>C5AR2</i>	0.7014**	—
<i>TLR4</i>	0.6125**	0.4722**
<i>ITGAM</i>	0.3018*	0.3689*
<i>COX2</i>	0.4662**	0.4377*
<i>CXCL8</i>	0.7016**	0.6774**

¹Correlation coefficient between C5aR and toll-like receptor (TLR)-4 related genes.

* $P < 0.05$ (2-tailed); ** $P < 0.01$ (2-tailed).

Protein Concentration. Our results show that the overall protein concentration was significantly lower (58%) in neutrophils obtained from periparturient cows ($P = 0.0007$) compared with those obtained from mid-lactation cows (Figure 4A, left column). This was also seen after incubation. In periparturient cows, we found 59% ($P = 0.016$), 56% ($P = 0.0008$), 57% ($P = 0.0009$), and 60% ($P = 0.005$) less protein in unstimulated (control) and stimulated (C5a, LPS, and C5a + LPS) cells, respectively (Figure 4B, right column). Although this finding is worth further investigation, it falls beyond the scope of our study. However, it is important for the interpretation of the caspase-3 assay, as this assay is based on fixed protein aliquots. Protein contents are markedly lower during the periparturient period, and this variation was taken into account when expressing and interpreting the results of functional assays (see below) based on protein content.

MPO Assay. Compared with mid-lactation cows, neutrophils obtained from periparturient cows showed an overall lower MPO chlorination activity after 2 h of incubation ($P = 0.0024$; Figure 4B, left column). In particular, a significant difference between the lactation stages was observed after stimulation with C5a ($P = 0.027$), with a lower MPO activity in periparturient cows (Figure 4B, right column).

Caspase Activity. Overall caspase-3 activity, measured per 50 μ g of protein extract of the neutrophils, showed a very significant effect for lactation stage ($P = 0.0002$; Figure 4C, left column). Overall, neutrophils obtained from periparturient cows showed less caspase-3 activity compared with the mid-lactation group. When investigating the separate stimuli, a significant difference between the lactation groups was found in nonstimulated cells ($P = 0.0006$) and in cells stimulated with C5a ($P = 0.0018$), LPS ($P = 0.0038$), and C5a + LPS ($P = 0.0048$), with a lower caspase-3 activity in neutrophils obtained from periparturient cows (Figure 4C, right column). As protein content is lower in periparturient cows, these differences are enhanced even if compared at the neutrophil level instead of the protein level.

IL-8 Production. Stimulation of the cells had similar effects on the production of IL-8 in both lactation groups. No significant effect of lactation stage was observed for the release of IL-8 in the culture medium. No interaction was found for stimulation and lactation stage for the production of IL-8. Neutrophils of both lactation stages thus reacted similar upon stimulation (Figure 4D).

Plasma Cortisol Concentration. Plasma cortisol concentrations were similar between both groups (19 and 18 nm/mL for periparturient and mid-lactation cows, respectively).

DISCUSSION

Despite the striking results obtained during the past 2 decades on neutrophil function in periparturient cattle, it is still not clear what links neutrophils' altered phenotype with the increased risk for periparturient diseases. Some functions are obviously decreased, such as chemotaxis and MPO activity, whereas other functions give contradictory results or are even increased during the periparturient period, such as phagocytosis

Figure 3. The *C5AR1/C5AR2* mRNA expression ratio in neutrophils after 2 h of stimulation. The first column denotes the effect of lactation stage, and the second column denotes the effect of lactation stage per stimulus. No stimulation was used as control. Control: no stimulus (Hanks' balanced salt solution); C5a: 50 ng/mL; LPS: 10 μ g/mL; LPS+C5a: C5a (50 ng/mL) + LPS (10 μ g/mL). Light gray bars represent neutrophils obtained from periparturient cows, and dark gray bars represent neutrophils obtained from mid-lactation cows. Significant differences between periparturient and mid-lactation cows are denoted with asterisks (* $P < 0.05$). Error bars represent mean ratio $\pm$ SEM.

(Kehrli et al., 1989; Cai et al., 1994; Moreira da Silva et al., 1998; Hammon et al., 2006).

Neutrophil influx into the lumen of the gland is triggered by microbial components and chemoattractants such as LPS, IL-8, and C5a (Riollet et al., 2000). Given

their importance in acute and chronic inflammatory conditions resulting from overactivation or dysregulation of neutrophils, TLR and C5a receptor signaling are attractive therapeutic targets for the treatment and prevention of inflammatory conditions (Riedemann et

Figure 4. Differences between neutrophils obtained during the periparturient period and mid lactation after 2 h of stimulation. (A) Protein concentration (µg/mL); (B) myeloperoxidase (MPO) chlorination activity per 6.25×10^5 neutrophils (relative fluorescence units; RFU); (C) caspase-3 activity per 50 µg of protein (relative light units; RLU); and (D) IL-8 concentration (pg/mL). No stimulation was used as control. Control: no stimulus (Hanks' balanced salt solution); C5a: 50 ng/mL; LPS: 10 µg/mL; LPS+C5a: C5a (50 ng/mL) + LPS (10 µg/mL). The first column denotes the effect of lactation stage, and the second column denotes the effect of lactation stage per stimulus. As we were interested in the differences between the lactation stages, the effect of stimulation compared with nonstimulated cells is not discussed in this figure. Data are presented as means $\pm$ SEM. Light gray bars represent neutrophils obtained from periparturient cows, and dark gray bars represent neutrophils obtained from mid-lactation cows. Significant differences between periparturient and mid-lactation cows are denoted with asterisks (* P < 0.05; ** P < 0.01; *** P < 0.001).

al., 2003; Guo and Ward, 2005; Kanzler et al., 2007). Therefore, understanding the mechanisms modulating TLR activity and the C5a–C5a receptor interaction is of major interest. Changes in receptor expression may influence the clinical outcome after infection. We were interested in how lactation stage affects the expression of these receptors and their downstream signaling pathways. More specifically, we (1) focused on C5AR2 and its expression in both lactation stages and (2) investigated its role in TLR-4 and complement signaling in peripartum and mid-lactation cows.

C5AR2 Expression in Periparturient Cows

We found that the overall expression of *C5AR2* is lower in periparturient cows. To our knowledge, this is the first study evaluating the expression and function of neutrophil *C5AR2* in periparturient cows. Murine and human studies investigating the role of *C5AR2* by use of knockout models and monoclonal antibodies to block C5AR2 revealed an excessive inflammatory response upon stimulation with C5a and LPS (Gerard et al., 2005; Chen et al., 2007; Raby et al., 2011; Holst et al., 2012). Based on these findings, C5AR2 has been reported to function as a modulator of C5a-induced responses (Chen et al., 2007) by inhibiting C5aR functionality (Gerard et al., 2005; Raby et al., 2011). Murine neutrophils deficient in *C5AR2* also show impaired surface expression of Mac-1 (CD11b/CD18) and a reduced inflammatory cell infiltration in response to C5a, whereas C5a induces a strong Mac-1 induction in wild-type neutrophils. This suggests that C5AR2 is critical for optimal C5a-mediated cell infiltration, at least in certain in vivo settings (Chen et al., 2007). Remarkably, in our study the decrease in *C5AR2* expression in the periparturient cows revealed similar effects upon stimulation with C5a and LPS, though not to the same extent, as seen in C5AR2-knockout models where C5AR2 function is completely abolished. We found a decrease in *ITGAM* expression and a different response upon LPS stimulation in periparturient cows. This phenomenon may be part of the complex underlying mechanism determining clinical severity of mastitis in periparturient cows. Severity of disease is determined by a scoring system based on systemic clinical signs measured by rectal temperature, skin turgor, rumen motility, and behavior of the cow. Total scores allow the classification of cow as mild, moderate, or severe responders (Vangroenweghe et al., 2004b). Migration of neutrophils from the blood to the locus of infection is of major importance in host defense against *E. coli* mastitis (Hill et al., 1979; Hill, 1981; Burvenich et al., 1989). When neutrophils do not reach the locus of infection on time, the inflammatory reaction can get out of bal-

ance and a severe systemic inflammation might develop (Stevens et al., 2012). Our findings are discussed in detail below.

Role of TLR4 in C5AR2 Expression

Lipopolysaccharide is a strong activator of the TLR-4 pathway. It is a primary initiator of inflammation and is therefore of considerable interest for its potential signaling interactions with the C5a pathways. One indication for the existence of crosstalk between the TLR-4 and the C5a pathway comes from the fact that in both pathways mitogen-activated protein kinase intermediates are involved in signal transduction, which in turn activate nuclear factor- κ B and cytokine synthesis (Zhang et al., 2007). In our study, we observed a strong correlation between *C5AR1* and *TLR4* expression (Table 2). The importance of possible crosstalk between these pathways is illustrated by the fact that both LPS and C5a are able to induce a cytokine response, and this response can be synergistic (Holst et al., 2012). In addition to the positive effect of TLR-4 stimulation on C5aR signaling, it was reported that C5aR stimulation can alter TLR-4-mediated signaling (Raby et al., 2011; Holst et al., 2012). This TLR-4-induced response did not result from upregulation of the C5aR on the cell surface, as its expression was in fact lower after exposure to LPS. This decreased surface expression of C5aR was not reflected on the level of gene expression in our study (Figure 2B, right column) and thus probably reflected C5a-induced receptor internalization (Raby et al., 2011). Beside the fact that mRNA levels are not always consistent with protein expression, this might also be due to our short incubation time (2 h) compared with the study of Raby et al. (2011; 14 h). However, in the study by Raby et al., hypersensitivity after LPS stimulation appeared to result mainly from the negative modulation of the C5AR2 by TLR-4, which in turn acted as an inhibitor of C5aR. Hence, TLR-4 activation probably enhances proinflammatory responses to C5a by “inhibiting the inhibitor” (Holst et al., 2012). Our data support this hypothesis: after stimulation with LPS, we observed a decrease in *C5AR2* expression for both periparturient and mid-lactation cows. This effect was especially seen in periparturient cows, resulting in a significant difference in the expression of *C5AR2* between both lactation stages (Figure 2E, right column). The positive effect of TLR-4 activation on C5a-mediated responses has obvious relevance for the overwhelming cytokine response that is a feature of both sepsis and sterile inflammation (Holst et al., 2012). Both C5a receptors show a lower expression in the periparturient cows, although this decreased expression is significant only for

C5AR2 (Figure 2B, E, left column). However, given the importance of a balanced C5aR/C5AR2 response, we analyzed the *C5AR1:C5AR2* mRNA expression ratio (Figure 3, left) and found a relative higher expression of *C5AR1* compared with *C5AR2* in periparturient cows. We believe that this altered expression ratio could also contribute to the cytokine storm seen in severe periparturient *E. coli* mastitis cases (Stevens et al., 2012). Stimulation with LPS (Figure 3, right) especially seems to induce differences in *C5AR1:C5AR2* expression ratio between the 2 lactation stages. This might be of importance, as relative lower expression of *C5AR2* during the periparturient period can enhance the proinflammatory function of *C5AR1*. Possibly, this increased ratio contributes to the shift toward proinflammation and subsequent excessive reaction often observed in mastitis cases where LPS is involved.

Effect of *C5AR2* Downregulation on Neutrophil Migration

Severe responders are characterized by a delayed recruitment of neutrophils to the place of infection (Hill et al., 1979; Hill, 1981; Burvenich et al., 1989). In our study, the most striking differences between both lactation stages were found in the expression of *ITGAM* (Figure 2C). For neutrophils, Mac-1 (CD11b/CD18) is one of the most important β_2 -integrins (Van Werven et al., 1997). Mac-1 is normally enhanced when neutrophils are activated: stimulation with LPS causes an upregulation of CD11b/CD18 (Lynn et al., 1991). Furthermore, previous studies also reported an increased expression of *ITGAM* after stimulation with C5a in an in vitro model (Mollnes et al., 2002). In the present study, stimulation with both C5a and LPS indeed resulted in an increase at the gene expression level of *ITGAM* in the neutrophils obtained from mid-lactation cows. In contrast, our results indicate that LPS even seems to have a negative effect on *ITGAM* expression in the periparturient group compared with nonstimulated cells (Figure 2C, right column), resulting in a very significantly different expression compared with the mid-lactation period. This is not in agreement with a previous study evaluating the expression of CD11b on the neutrophil cell surface in periparturient cows. Diez-Fraile et al. (2003a, b) found that CD11b was enhanced upon stimulation with LPS. However, the analysis of CD11b expression is restricted to the periparturient (up until 5 wk after parturition) period in these studies, and no comparison was made between neutrophils obtained at the day of parturition and those obtained from mid lactation. In addition, CD11b is prepacked in secretory vesicles (Faurischou and Borregaard, 2003), and its mobilization to the plasma membrane upon

stimulation might not reflect the underlying gene expression. Especially for neutrophils undergoing differentiation and during response to inflammatory stimuli, an inconsistent correlation between mRNA and protein expression levels has been observed (Fessler et al., 2002). Instead of the expected upregulation of *ITGAM*, periparturient cows thus demonstrate a downregulation of this receptor upon LPS stimulation. Our findings support the observations of a study by Kremer et al. (1993) in which chemotaxis during experimental *E. coli* in periparturient cows (3–6 wk after calving) revealed a decreased chemotaxis after inoculation. In addition, a higher chemotactic responsiveness was found in neutrophils in moderate responders compared with severe responders both before and during experimental *E. coli* mastitis. The importance of these observations lies in the necessity of fast and correct localization of neutrophils to eradicate the invaded pathogens. An explanation for the reduced neutrophil recruitment in periparturient cows might be found in the decreased expression of *C5AR2*. Deficiency of *C5AR2*, as shown by an in vitro study with *C5AR2* $-/-$ mice, results in reduced inflammatory cell infiltration. Neutrophils of these *C5AR2* knockout mice showed reduced Mac-1 (CD11b/CD18) expression compared with wild type mice (Chen et al., 2007). These authors hypothesized that the C5AR2 receptor is important for the infiltration capacity of neutrophils. In our study, we observed that the expression of *C5AR2* and *ITGAM* is indeed positively correlated (Table 2). An eventual dependence of *ITGAM* expression on the expression of *C5AR2* might explain the slower infiltration of neutrophils into the mammary gland during the periparturient period because (1) a lower expression of *C5AR2* might result in less expression of *ITGAM* compared with mid lactation and (2) stimulation of TLR-4 by LPS further decreases the C5AR2 functionality. This could offer an explanation for the delayed recruitment of neutrophils in severe periparturient *E. coli* mastitis (Vandeputte-Van Messom et al., 1993), where LPS is involved in the inflammatory reaction.

Effect of *C5R2* Downregulation on Viability

A second characteristic of severe responders during the periparturient period is prolonged neutrophil survival—an effect that is attributed to high levels of C5a (Perianayagam et al., 2002; Burton et al., 2005). Tight regulation of neutrophil apoptosis is a key feature of normal neutrophil function. Spontaneous apoptosis maintains homeostasis but also regulates efficient resolution of inflammation (Lakschevitz et al., 2015). Our results indicate a decrease in spontaneous apoptosis, as determined by caspase-3 activity, during the peri-

parturient period (Figure 4). Caspase-3 is a key factor in apoptosis execution (Thornberry, 1998). Supporting evidence of this change in neutrophil apoptosis was also reported on the gene expression level by Madsen et al. (2002) and Crookenden et al. (2016). They reported a decreased expression in several proapoptotic genes with a concomitant increase in the expression of antiapoptotic genes on the day of parturition. A possible explanation for the difference in viability between both lactation stages is that parturition primes the neutrophils, by which they get more sensitive to inflammatory stimuli. Stress, including parturition, increases the release of neutrophils from the bone marrow, followed by an increase in immature (band) cells in the circulation (Crookenden et al., 2016). Although our isolation protocol is set up to isolate mature neutrophils, a considerable percentage (25%) of the isolated neutrophils obtained from periparturient cows are band neutrophils. To a certain extent, these band neutrophils might also contribute to the increased viability observed in periparturient cells, which might be linked to the high cortisol levels at parturition (Burton et al., 1995; Crookenden et al., 2016). Although a shift in neutrophil population during the periparturient period could provide an explanation for the prolonged viability, the question remains whether the increased viability is solely due to this shift. Evidence suggests a role of C5AR2 regulation of viability. A first clue is found in the functionality of MPO, which is discussed below.

Effect of C5R2 Downregulation on the Oxidative Burst

The azurophilic enzyme MPO is a third potential player in the increased severity seen after parturition. We found an overall lower activity for MPO in periparturient cows (Figure 4B, left column). Our results confirm the known lower activity level of MPO during the periparturient period (Kehrli et al., 1989). Interestingly, the largest difference between periparturient and mid-lactation cows is found after stimulation with C5a, resulting in a significant difference between both lactation stages (Figure 4B, right column). Mollnes et al. (2002) reported an essential role for both C5a and C5aR in *E. coli*-induced MPO release, as both inhibiting C5a formation and blocking the C5aR abolished the release of MPO. The role of C5AR2 was not investigated by Mollnes et al. (2002). In addition, caution must be taken when interpreting our results compared with the *E. coli* study, as it is unlikely that LPS exhibits the exact same effects of *E. coli* in MPO expression and function. We decided to use LPS for stimulation to give a similar treatment to the neutrophils.

A link between periparturient susceptibility for severe infection, C5AR2 downregulation, and decreased MPO functionality might be found in the broad functionality of MPO. In addition to its antibacterial functions, growing evidence suggests that MPO is essential for neutrophil shutdown and thereby timely resolution of inflammation (El Kebir and Filep, 2013). Recent studies indicated that diminished MPO expression might be a good predictor for identifying septic shock patients at high risk of death and that MPO plays, at least in part, a protective role during endotoxemia. This might also explain the severe cases of mastitis typically occurring during the periparturient period. Blockade or genetic deletion of MPO resulted in increased hyperthermia and mortality after LPS challenge in a mouse model (Reber et al., 2017). One proposed mechanism by which MPO is protective may be signaling through β_2 -integrins in neutrophils, thereby regulating apoptosis (review by El Kebir and Filep, 2013). Myeloperoxidase is released during phagocytosis (Cooray et al., 1993). Neutrophils can integrate these signals through a common downstream mechanism, which results in contrasting survival or death decision in neutrophils. Engagement of CD11b/CD18 with MPO delays apoptosis by generating survival cues through activation of the downstream PI3K (phosphatidylinositol 3-kinase)/Akt (protein kinase B) and MEK (MAPK or ERK kinase)/ERK (extracellular signal-regulated kinase) pathways that inhibit the caspase pathways. On the other hand, CD11b/CD18-mediated phagocytosis of opsonized bacteria, such as *E. coli*, evokes reactive oxygen species production by NADPH oxidase, leading to mediated activation of caspase-8 and acceleration of the cell death program. However, not all stimuli that generate reactive oxygen species trigger neutrophil death. The amount, nature, and localization of release likely determines their proapoptotic potential. During sepsis, neutrophils are reported to show delayed apoptosis (Riedemann et al., 2003). The latter phenomenon may result in enhanced tissue damage due to persistence of neutrophils. As C5AR2 seems necessary for appropriate CD11b function, more research into the role of C5AR2, including the effect of stimulation with both LPS and *E. coli* on MPO functionality, might lead to therapeutic possibilities to treat these severe inflammatory cases (El Kebir and Filep, 2013).

Protein Concentration in Periparturient Cows

Attention should be paid to the interpretation of the caspase-3 data. Caspase-3 activity was determined per 50 μ g of protein, as described by the manufacturer's protocol. In the present study, however, protein concentration in neutrophils from the periparturient cows was

much lower than that in mid-lactation cows (Figure 4A). Ideally, caspase-3 activity would have been analyzed on the same amount of neutrophils, just like the other functional tests in the present study. The detection kit, however, did not include standards. While analyzing our data, we decided to re-evaluate protein concentration in both lactation stages and included the same caspase-3 assay—this time with standards—in our analysis. We tested it on a new population of periparturient and mid-lactation cows with exactly the same conditions. The results of this extra test confirmed the findings of the present study, reassuring us that our findings were not merely due to coincidence. We also analyzed caspase-3 activity per 2.5×10^6 neutrophils (instead of per 50 μg of protein) and as we expected found an even more increased difference between periparturient and mid-lactation cows. To our knowledge, we are the first to demonstrate such differences in protein content between periparturient and mid-lactation cows. Previously, variations in protein concentration have been correlated with both the cell's position in the cell cycle and cell volume (Erlinger and Saier, 1982; Cookson et al., 2010). Even cell volume of individual cells within a cell population can vary, independently of their cell cycle phase (Crissman and Steinkamp, 1973; Tzur et al., 2009; Bryan et al., 2014). We did not investigate cellular volume in the present study, but this might require further investigation. As biochemical reactions depend on the concentration of reactants and enzymes, the absolute numbers of these molecules would have to scale with cellular volume (Padovan-Merhar et al., 2015). Thus, ignoring volume fluctuation can lead to incomplete conclusions regarding protein concentration.

CONCLUSIONS

Collectively, our results shed light on the complex role of C5AR2 during periparturient *E. coli* mastitis. First, expression of C5AR2, and thus its anti-inflammatory function, is decreased during parturition. Its downregulation and the balance between C5aR and C5AR2 expression, observed by the C5aR/C5AR2 mRNA expression ratio, might contribute to the excessive proinflammatory reaction often observed during periparturient mastitis. Moreover, our data suggest the existence of a crosstalk between the C5aR and TLR-4 signaling pathway in these cells based on the correlations between the receptor genes. The most striking observation was the lower expression of C5AR2 and ITGAM after stimulation with LPS in periparturient cows compared with mid-lactation cows. Large amounts of LPS can be released in the infected mammary glands of cows with naturally occurring *E. coli* mastitis. *Escherichia coli* is still an important agent causing clinical

mastitis in dairy cows (Botrel et al., 2010). Our results indicate that infection with *E. coli* might magnify the differences in immune competence between periparturient and mid-lactation cows and even result in a different response of the periparturient cows upon infection. More specifically, neutrophils from periparturient cows produce less anti-inflammatory signals to balance the reaction and show an increased risk of improper localization. As MPO has a role in activating other neighboring neutrophils, supporting cell adhesion via interaction with CD11b/CD18 integrins, and regulating apoptosis, more research should be done to further elucidate the central role of MPO in inflammation during the periparturient period. This is especially the case if LPS is involved, as LPS probably plays a role in the regulation of neutrophil adhesion receptor expression during mastitis.

ACKNOWLEDGMENTS

We thank Jo Vandesompele (Department of Pediatrics and Medical Genetics, Ghent University, Ghent, Belgium) for the helpful discussion and suggestions.

REFERENCES

- Adrover, J. M., J. A. Nicolás-Ávila, and A. Hidalgo. 2016. Aging: A temporal dimension for neutrophils. *Trends Immunol.* 37:334–345. <https://doi.org/10.1016/j.it.2016.03.005>.
- Botrel, M. A., M. Haenni, E. Morignat, P. Sulpice, J. Y. Madec, and D. Calavas. 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Alpes, France. *Foodborne Pathog. Dis.* 7:479–487. <https://doi.org/10.1089/fpd.2009.0425>.
- Bryan, A. K., V. C. Hecht, W. Shen, K. Payer, W. H. Grover, and S. R. Manalis. 2014. Measuring single cell mass, volume, and density with dual suspended microchannel resonators. *Lab Chip* 14:569–576. <https://doi.org/10.1039/c3lc51022k>.
- Burton, J. L., M. E. Kehrli, S. Kapil, and R. L. Horst. 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: Effects of cortisol and dexamethasone. *J. Leukoc. Biol.* 57:317–325.
- Burton, J. L., S. A. Madsen, L. C. Chang, P. S. Weber, K. R. Buckham, R. van Dorp, M. Hickey, and B. Earley. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain “neutrophil dysfunction” in parturient dairy cows. *Vet. Immunol. Immunopathol.* 105:197–219. <https://doi.org/10.1016/j.vetimm.2005.02.012>.
- Burvenich, C., R. Heyneman, G. VandePutte-van Messom, A. M. Massart-Leën, and E. Roets. 1989. Differences in local and systemic reactions during severe and moderate cases of *Escherichia coli* mastitis in cattle soon after parturition. Page 20 in *Proc. Int. Conf. Mastitis*, Vienna, Austria.
- Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet. Res.* 34:521–564. <https://doi.org/10.1051/vetres:2003023>.
- Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611–622. <https://doi.org/10.1373/clinchem.2008.112797>.

- Cai, T. Q., P. G. Weston, L. A. Lund, B. Brodie, D. J. McKenna, and W. C. Wagner. 1994. Association between neutrophil functions and periparturient disorders in cows. *Am. J. Vet. Res.* 55:934–943.
- Chen, N. J., C. Mirtsos, D. Suh, Y. C. Lu, W. J. Lin, C. McKelvie, T. Lee, H. Baribault, and W. C. Yeh. 2007. C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a. *Nature* 446:203–207. <https://doi.org/10.1038/nature05559>.
- Cookson, N. A., S. W. Cookson, L. S. Tsimring, and J. Hasty. 2010. Cell cycle-dependent variations in protein concentration. *Nucleic Acids Res.* 38:2676–2681. <https://doi.org/10.1093/nar/gkp1069>.
- Cooray, R., C. B. G. Peterson, and O. Holmberg. 1993. Isolation and purification of bovine myeloperoxidase from neutrophil granules. *Vet. Immunol. Immunopathol.* 38:261–272.
- Crissman, H. A., and J. A. Steinkamp. 1973. Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J. Cell Biol.* 59:766–771.
- Crookenden, M. A., A. Heiser, A. Murray, V. S. R. Dukkupati, J. K. Kay, J. J. Loo, S. Meier, M. D. Mitchell, K. M. Moyes, C. G. Walker, and J. R. Roche. 2016. Parturition in dairy cows temporarily alters the expression of genes in circulating neutrophils. *J. Dairy Sci.* 99:6470–6483. <https://doi.org/10.3168/jds.2015-10877>.
- Diez-Fraile, A., L. Duchateau, E. Meyer, and C. Burvenich. 2003a. Expression of β_2 -integrin on monocytes and blood polymorphonuclear leukocytes in the periparturient period in dairy cows. *Can. J. Vet. Res.* 67:235–238.
- Diez-Fraile, A., E. Meyer, L. Duchateau, and C. Burvenich. 2003b. L-Selectin and β_2 -Integrin expression on circulating bovine polymorphonuclear leukocytes during endotoxin mastitis. *J. Dairy Sci.* 86:2334–2342.
- El Kebir, D., and J. G. Filep. 2013. Modulation of neutrophil apoptosis and the resolution of inflammation through β_2 -integrins. *Front. Immunol.* 4:60. <https://doi.org/10.3389/fimmu.2013.00060>.
- Erlinger, S. U., and M. H. Saier Jr.. 1982. Decrease in protein content and cell volume of cultured dog kidney epithelial cells during growth: Importance for transport measurements. *In Vitro* 18:196–202.
- Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* 5:1317–1327. <https://doi.org/10.1016/j.micinf.2003.09.008>.
- Fessler, M. B., K. C. Malcolm, M. W. Duncan, and G. S. Worthen. 2002. Lipopolysaccharide stimulation of the human neutrophil. An analysis of changes in gene transcription and protein expression by oligonucleotide microarrays and proteomics. *Chest* 121(Suppl.):75S–76S.
- Gerard, N. P., B. Lu, P. Liu, S. Craig, Y. Fujiwara, S. Okinaga, and C. Gerard. 2005. An anti-inflammatory function for the complement anaphylatoxin C5a-binding protein, C5L2. *J. Biol. Chem.* 280:39677–39680. <https://doi.org/10.1074/jbc.C500287200>.
- Gilbert, R. O., Y. T. Grohn, P. M. Miller, and D. J. Hoffman. 1993. Effect of parity on periparturient neutrophil function in dairy cows. *Vet. Immunol. Immunopathol.* 36:75–82.
- Goossens, K., M. Van Poucke, A. Van Soom, J. Vandesompele, A. Van Zeven, and L. Peelman. 2005. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Dev. Biol.* 5:27. <https://doi.org/10.1186/1471-213X-5-27>.
- Guo, R. F., and P. A. Ward. 2005. Role of C5a in inflammatory responses. *Annu. Rev. Immunol.* 23:821–852. <https://doi.org/10.1146/annurev.immunol.23.021704.115835>.
- Hammon, D. S., I. M. Evjen, T. R. Dhiman, J. P. Goff, and J. L. Walters. 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. *Vet. Immunol. Immunopathol.* 113:21–29. <https://doi.org/10.1016/j.vetimm.2006.03.022>.
- Hellemans, J., and J. Vandesompele. 2011. qPCR data analysis: Unlocking the secret to successful results. Pages 139–150 in *PCR Troubleshooting and Optimization: The Essential Guide*. S. Kennedy and N. Oswald, ed. Caister Academic Press, Poole, UK.
- Hill, A. W. 1981. Factors influencing the outcome of *Escherichia coli* mastitis in the dairy cow. *Res. Vet. Sci.* 31:107–112.
- Hill, A. W., A. L. Shears, and K. G. Hibbitt. 1979. The pathogenesis of experimental *Escherichia coli* mastitis in newly calving cows. *Res. Vet. Sci.* 26:97–101.
- Holst, B., A. C. Raby, J. E. Hall, and M. O. Labéta. 2012. Complement takes its Toll: An inflammatory crosstalk between Toll-like receptors and the receptors for the complement anaphylatoxin C5a. *Anaesthesia* 67:60–64. <https://doi.org/10.1111/j.1365-2044.2011.07011.x>.
- Kanzler, H., F. J. Barrat, E. M. Hessel, and R. L. Coffman. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* 13:552–559. <https://doi.org/10.1038/nm1589>.
- Kehrli, M. E., B. J. Nonnecke, and J. A. Roth. 1989. Alterations in bovine neutrophil function during the periparturient period. *Am. J. Vet. Res.* 50:207–214.
- Kremer, W. D. J., E. N. Noordhuizen-Stassen, F. J. Grommers, A. J. J. M. Daemen, and A. Brand. 1993. Blood polymorphonuclear leukocyte chemotaxis during experimental *Escherichia coli* bovine mastitis. *J. Dairy Sci.* 76:2613–2618.
- Lakschevitz, F. S., M. B. Visser, C. Sun, and M. Glogauer. 2015. Neutrophil transcriptional profile changes during transit from bone marrow to sites of inflammation. *Cell. Mol. Immunol.* 12:53–65. <https://doi.org/10.1038/cmi.2014.37>.
- Li, R., L. G. Coulthard, M. C. Wu, S. M. Taylor, and T. M. Woodruff. 2013. C5L2: A controversial receptor of complement anaphylatoxin, C5a. *FASEB J.* 27:855–864. <https://doi.org/10.1096/fj.12-220509>.
- Lynn, W. A., C. R. Raetz, N. Qureshi, and D. T. Golenbock. 1991. Lipopolysaccharide-induced stimulation of CD11b/CD18 expression on neutrophils. Evidence of specific receptor-based response and inhibition by lipid A-based antagonists. *J. Immunol.* 147:3072–3079.
- Madsen, S. A., P. S. Weber, and J. L. Burton. 2002. Altered expression of cellular genes in neutrophils of periparturient dairy cows. *Vet. Immunol. Immunopathol.* 86:159–175. [https://doi.org/10.1016/S0165-2427\(02\)00020-X](https://doi.org/10.1016/S0165-2427(02)00020-X).
- Mollnes, T. E., O. L. Brekke, M. Fung, H. Fure, D. Christiansen, G. Bergseth, V. Videm, K. T. Lappegård, J. Köhl, and J. D. Lambris. 2002. Essential role of the C5a receptor in *E. coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100:1869–1877.
- Moreira da Silva, F., C. Burvenich, A. M. Massart Leën, and L. Brosse. 1998. Assessment of blood neutrophil oxidative burst activity in dairy cows during the period of parturition. *Anim. Sci.* 67:421–426. <https://doi.org/10.1017/S1357729800032823>.
- Nicholson, D. W. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* 6:1028–1042. <https://doi.org/10.1038/sj.cdd.4400598>.
- Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. *Vet. Res.* 34:597–627. <https://doi.org/10.1051/vetres:2003024>.
- Paape, M. J., E. M. Lilius, P. A. Wiitanen, M. P. Kontio, and R. H. Miller. 1996. Intramammary defense against infections induced by *Escherichia coli* in cows. *Am. J. Vet. Res.* 57:477–482.
- Padovan-Merhar, O., G. P. Nair, A. Bialesch, A. Mayer, S. Scarfone, S. W. Foley, A. R. Wu, L. S. Churchman, A. Singh, and A. Raj. 2015. Single mammalian cells compensate for differences in cellular volume and DNA copy number through independent global transcriptional mechanisms. *Mol. Cell* 58:339–352. <https://doi.org/10.1016/j.molcel.2015.03.005>.
- Perianayagam, M. C., V. S. Balakrishnan, A. J. King, B. J. Pereira, and B. L. Jaber. 2002. C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway. *Kidney Int.* 61:456–463. <https://doi.org/10.1046/j.1523-1755.2002.00139.x>.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. <https://doi.org/10.1093/nar/29.9.e45>.
- Raby, A. C., B. Holst, J. Davies, C. Colmont, Y. Laumonnier, B. Coles, S. Shah, J. Hall, N. Topley, J. Köhl, B. P. Morgan, and M. O. Labeta. 2011. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. *Eur. J. Immunol.* 41:2741–2752. <https://doi.org/10.1002/eji.201041350>.

- Reber, L. L., C. M. Gillis, P. Starkl, F. Jonsson, R. Sibilano, T. Marichal, N. Gaudenzio, M. Bérard, S. Rogella, C. H. Contag, P. Bruhns, and S. J. Galli. 2017. Neutrophil myeloperoxidase diminishes the toxic effects and mortality induced by lipopolysaccharide. *J. Exp. Med.* 214:1249–1258. <https://doi.org/10.1084/jem.20161238>.
- Revelo, X. S., and M. R. Waldron. 2012. In vitro effects of *Escherichia coli* lipopolysaccharide on the function and gene expression of neutrophils isolated from the blood of dairy cows. *J. Dairy Sci.* 95:2422–2441. <https://doi.org/10.3168/jds.2011-4616>.
- Riedemann, N. C., R. F. Guo, and P. A. Ward. 2003. Novel strategies for the treatment of sepsis. *Nat. Med.* 9:517–524. <https://doi.org/10.1038/nm0503-517>.
- Riollet, C., P. Rainard, and B. Poutrel. 2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin. Diagn. Lab. Immunol.* 7:161–167. <https://doi.org/10.1128/CDLI.7.2.161-167.2000>.
- Shuster, D. E., M. E. Jr. Kehrli, P. Rainard, and M. Paape. 1997. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. *Infect. Immun.* 65:3286–3292.
- Siemsen, D. W., I. A. Schepetkin, L. N. Kirpotina, B. Lei, and M. T. Quinn. 2007. Neutrophil isolation from nonhuman species. *Methods Mol. Biol.* 412:21–34. https://doi.org/10.1007/978-1-59745-467-4_3.
- Smits, E., E. Cifrian, A. J. Guidry, P. Rainard, C. Burvenich, and M. J. Paape. 1996. Cell culture system for studying bovine neutrophil diapedesis. *J. Dairy Sci.* 79:1353–1360.
- Stevens, M. G., B. De Spiegeleer, L. Peelman, X. Boulougouris, A. V. Capuco, and C. Burvenich. 2012. Compromised neutrophil function and bovine *E. coli* mastitis: Is C5a the missing link? *Vet. Immunol. Immunopathol.* 149:151–156. <https://doi.org/10.1016/j.vetimm.2012.07.002>.
- Stevens, M. G., M. Van Poucke, L. Peelman, P. Rainard, B. De Spiegeleer, C. Rogiers, G. R. Van De Walle, L. Duchateau, and C. Burvenich. 2011b. Anaphylatoxin C5a-induced Toll-like receptor 4 signaling in bovine neutrophils. *J. Dairy Sci.* 94:152–164. <https://doi.org/10.3168/jds.2010-3358>.
- Stevens, M. G. H., L. J. Peelman, B. De Spiegeleer, A. Pezeshki, G. R. Van De Walle, L. Duchateau, and C. Burvenich. 2011a. Differential gene expression of the toll-like receptor-4 cascade and neutrophil function in early- and mid-lactating dairy cows. *J. Dairy Sci.* 94:1277–1288. <https://doi.org/10.3168/jds.2010-3563>.
- Thornberry, N. A. 1998. Caspases: Key mediators of apoptosis. *Chem. Biol.* 5:R97–R103.
- Tzur, A., R. Kafri, V. S. LeBleu, G. Lahav, and M. W. Kirschne. 2009. Cell growth and size homeostasis in proliferating animal cells. *Science* 325:167–171. <https://doi.org/10.1126/science.1174294>.
- Van Werven, T., E. N. Noordhuizen-Stassen, A. J. Daemen, Y. H. Schukken, A. Brand, and C. Burvenich. 1997. Preinfection *in vitro* chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. *J. Dairy Sci.* 80:67–74.
- Vandeputte-Van Messom, G., C. Burvenich, E. Roets, A. M. Massart-Leën, R. Heyneman, W. D. Kremer, and A. Brand. 1993. Classification of newly calved cows into moderate and severe responders to experimentally induced *Escherichia coli* mastitis. *J. Dairy Res.* 60:19–29.
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.
- Vangroenweghe, F., L. Duchateau, and C. Burvenich. 2004a. Moderate inflammatory reaction during experimental *Escherichia coli* mastitis in primiparous cows. *J. Dairy Sci.* 87:886–895. [https://doi.org/10.3168/jds.S0022-0302\(04\)73233-6](https://doi.org/10.3168/jds.S0022-0302(04)73233-6).
- Vangroenweghe, F., P. Rainard, M. Paape, L. Duchateau, and C. Burvenich. 2004b. Increase of *Escherichia coli* inoculum doses induces faster innate immune response in primiparous cows. *J. Dairy Sci.* 87:4132–4144.
- Zhang, X., Y. Kimura, C. Fang, L. Zhou, G. Sfyroera, J. D. Lambris, R. A. Wetsel, T. Miwa, and W. C. Song. 2007. Regulation of Toll-like receptor-mediated inflammatory response by complement *in vivo*. *Blood* 110:228–236. <https://doi.org/10.1182/blood-2006-12-063636>.