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Effector responses of bovine blood neutrophils against *Escherichia coli*: Role of NOD1/NF-κB signalling pathway

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ABSTRACT

Neutrophils use a broad array of pattern recognition receptors to sense and respond to invading pathogens and are important in the early control of acute bacterial infections. Nucleotide-binding oligomerizing domain-1 (NOD1) is a cytoplasmic receptor involved in recognizing bacterial peptidoglycan. Reduced neutrophil NOD1 expression has been reported in periparturient dairy cows. The aim of this study was to investigate the role of NOD1 signalling in the early responses of bovine neutrophils to bacterial infections. Blood neutrophils from healthy heifers were preincubated for 2 h with ML130, a selective inhibitor of NOD1-dependent nuclear factor-κB (NF-κB) activation. Thereafter, cells were cultured with live *Escherichia coli* for additional 30 min or subjected to Boyden chamber cell migration assay with *E. coli* in the lower chamber. Results showed that ML130 inhibited *E. coli*-induced NF-κB nuclear translocation. There was an indication, although not significant, that ML130 down-regulated gene expression of proinflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, chemokines IL-8 and C-X-C motif ligand 2 (CXCL2), and adhesion molecule CD62L, in *E. coli*-challenged neutrophils. Flow cytometry-based Annexin V staining revealed a considerable increase in neutrophil survival upon *E. coli* infection, an effect that was attenuated in the presence of ML130. Additionally, inhibition of NOD1/NF-κB signalling resulted in reduced migration of neutrophils to *E. coli*, and impaired phagocytosis, intracellular bacterial killing and reactive oxygen species production by neutrophils. These results indicate that NOD1/NF-κB pathway plays a crucial role in modulating neutrophil responses that are important for early control of infections. Approaches aiming at restoring neutrophil NOD1 function could be beneficial for prevention or treatment of coliform mastitis.

1. Introduction

Neutrophils, as the most abundant professional phagocytes, provide the first line of defense against infections through their capacity to phagocytise and kill invading pathogens (Segal, 2005). In addition to being professional phagocytes, neutrophils are able to release numerous cytokines and chemokines that orchestrate the early host defense against infections (Mantovani et al., 2011; Parker et al., 2005).

The importance of neutrophils in protecting bovine mammary gland from infectious diseases has long been a crucial concern (Burvenich et al., 2004; Paape et al., 2003; Stevens et al., 2012). It is thought that defense of mammary gland is only effective if rapid influx of neutrophils from the circulation and subsequent phagocytosis and killing of bacteria occur (Paape et al., 2002). Mounting evidence indicates that neutrophil dysfunction contributes to the increased incidence of mastitis around parturition and during early lactation (Burvenich et al., 2007). However, *mechanisms underlying neutrophil dysfunction remain to be elucidated.*

Recognition of bacterial components by host-specific molecules is the first step in the defense against invading bacteria. Detection of pathogens by innate immune system is mediated by a variety of pattern recognition receptors (PRRs) (Thomas and Schroder, 2013), amongst which the nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs) have emerged as key microbial sensors (Chen et al., 2009; Clarke et al., 2010; Franchi et al., 2009). NOD1 and NOD2 proteins are the first mammalian members of the NLR family to be reported to act as intracellular sensors of peptidoglycan (PGN) moieties from Gram-negative and Gram-positive bacteria (Chamaillard et al., 2003; Girardin et al., 2003a,b; Inohara et al., 1999). Once activated, these receptors trigger intracellular signalling pathways that lead to the activation of transcriptional responses culminating in the expression of a subset of proinflammatory cytokines and chemokines, and activation of NOD1 and

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NOD2 induces neutrophil recruitment in vivo (Franchi et al., 2009; Frutuoso et al., 2010; Masumoto et al., 2006). Although NODs are initially thought to recognise intracellular microbes, it is becoming apparent that they play key roles in host immune defense against extracellular pathogens by sensing their components gaining access to the cytoplasm (Clarke and Weiser, 2011; Ratner et al., 2007).

One of the main outcomes of NOD1 and NOD2 activation by their respective agonists is the activation of nuclear factor-κB (NF-κB) (Hasegawa et al., 2008). Binding of NOD ligands results in the recruitment of serine-threonine kinase RICK (RIP-like interacting CLARP kinase) [also known as receptor-interacting protein 2 (Rip2)], followed by the activation of transforming growth factor β-activated kinase (TAK1) complex, polyubiquitination of inhibitor of NF-κB kinase (IKK)-β, degradation of the NF-κB repressor IκB, and the translocation of NF-κB to the nucleus (Moreira and Zamboni, 2012).

Both NOD1 and NOD2 have been recently identified in bovine neutrophils (Tan et al., 2012). Interestingly, in contrast to NOD2 that remained unchanged, NOD1 expression in blood neutrophils derived from periparturient dairy cows is dramatically reduced, leading to depressed neutrophil phagocytic activity and oxidative burst upon the stimulation of pure NOD1 agonist (Tan et al., 2012). However, as many pathogens express ligands of multiple PRRs (Kawai and Akira, 2011), whether impairment of NOD1 is sufficient to suppress the effector responses of bovine neutrophils to whole bacterial cells remains unknown, let alone the relevance of reduced neutrophil NOD1 to periparturient mastitis susceptibility.

Escherichia coli infection is the most common cause of mastitis, and the increased susceptibility and severity of this disease has been correlated with reduced neutrophil function during the periparturient period (Stevens et al., 2012). To ascertain a linkage to infectious diseases, we examined the dependence of cytokine/chemokine gene expression, cell death, migration, and the bacterial killing capacity on NOD1-mediated NF-κB activation in bovine neutrophils challenged with live *E. coli*.

2. Material and methods

2.1. Ethics statement

All procedures described below were approved by the Ethical Committee of Zhejiang University.

2.2. Escherichia coli strain

A reference strain *E. coli* ATCC2592 was provided by the Diagnostic Microbiology Laboratory at the Department of Veterinary Medicine, Zhejiang University. For experiments, bacteria were grown into log phase in Luria-Bertani (LB) broth at 37 °C. Next, bacteria were harvested, suspended, and washed three times in PBS, and plated on LB agar plates to determine the colony-forming units (CFU) inoculated in the following experiments.

2.3. Animal and blood sample collection

Peripheral blood samples were collected from the tail veins of Chinese Holstein heifers aged 8 to 9 months. Heifers were fed grass and corn silage and hay. All heifers were free of signs of illness such as depression, fever, in appetence, nasal discharge, abnormal feces, etc., on the day of sampling. Blood was collected into plastic tubes containing 10% by volume of acid citrate dextrose (ACD) anticoagulant and immediately transferred to laboratory.

2.4. Isolation of neutrophils

Neutrophils were isolated by centrifugation as previously described (Tan et al., 2012). Briefly, samples were centrifuged at 1000 × *g* for 20 minutes (min), and the serum and upper third of the packed red blood cells were discarded. Neutrophils were isolated from the remaining erythrocytes by adding hypotonic lysis buffer (5.5 mM NaH2PO4, 8.4 mM HK2PO4, pH 7.2) and isotonicity was regained by adding a hypertonic solution (5.5 mM NaH2PO4, 8.4 mM HK2PO4, 0.46 M NaCl, pH 7.2). All chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). After centrifugation (for 10 min at 600 × *g*), the resulting pellet was resuspended and washed twice with cold phosphate-buffered saline (PBS, pH 7.4). Neutrophils were shown to be 95% pure morphologically by microscopy. Viability was assessed by trypan blue dye exclusion and always exceeded 98%. Finally, cells were suspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS).

2.5. Cell treatments

Neutrophils were placed onto 24-well plates at 2×10^7 cells/well. The cells were preincubated with or without 30 μM ML130 (Selleckchem), a potent and selective inhibitor of NOD1-induced NF-κB activation (Khan et al., 2011), for 2 h (h) at 37 °C and 5% CO2. Live cells were then counted and diluted to 1×10^6 cells/well, and incubated in the presence or absence of 1×10^7 CFU *E. coli* for additional 30 min. Neutrophils in the suspension were separated from bacteria by centrifugation at 400 × *g* for 3 min, and subjected to Western blot, quantitative real-time PCR (qPCR), and apoptosis analyses.

2.6. Preparation of nuclear extracts and Western blot analysis

Nuclear extracts were prepared as described previously (Tamassia et al., 2007). Protein concentrations were determined by the Pierce BCA protein assay. Equal amount of proteins were separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, blocked overnight in blocking solution (10%

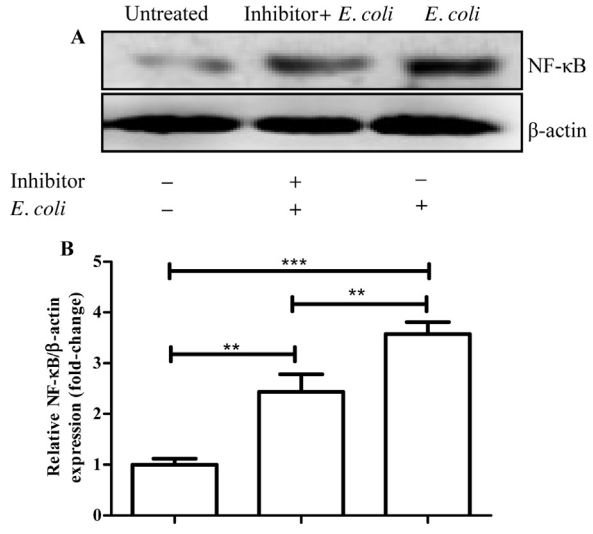

Fig. 1. Effect of ML130 on *E. coli*-induced NF-κB activation in neutrophils. (A) Freshly isolated neutrophils were pretreated with or without ML130 for 2 h, followed by exposure to live *E. coli* for 30 min. Nuclear translocation of NF-κB was assessed by Western blot analysis. (B) The densitometric analysis of Western blots. Data (mean ± SEM of 4 heifers) are presented as fold changes over untreated samples after normalisation to β-actin within the same sample. One of 3 independent experiments is shown. ** $P < 0.01$ and *** $P < 0.001$.

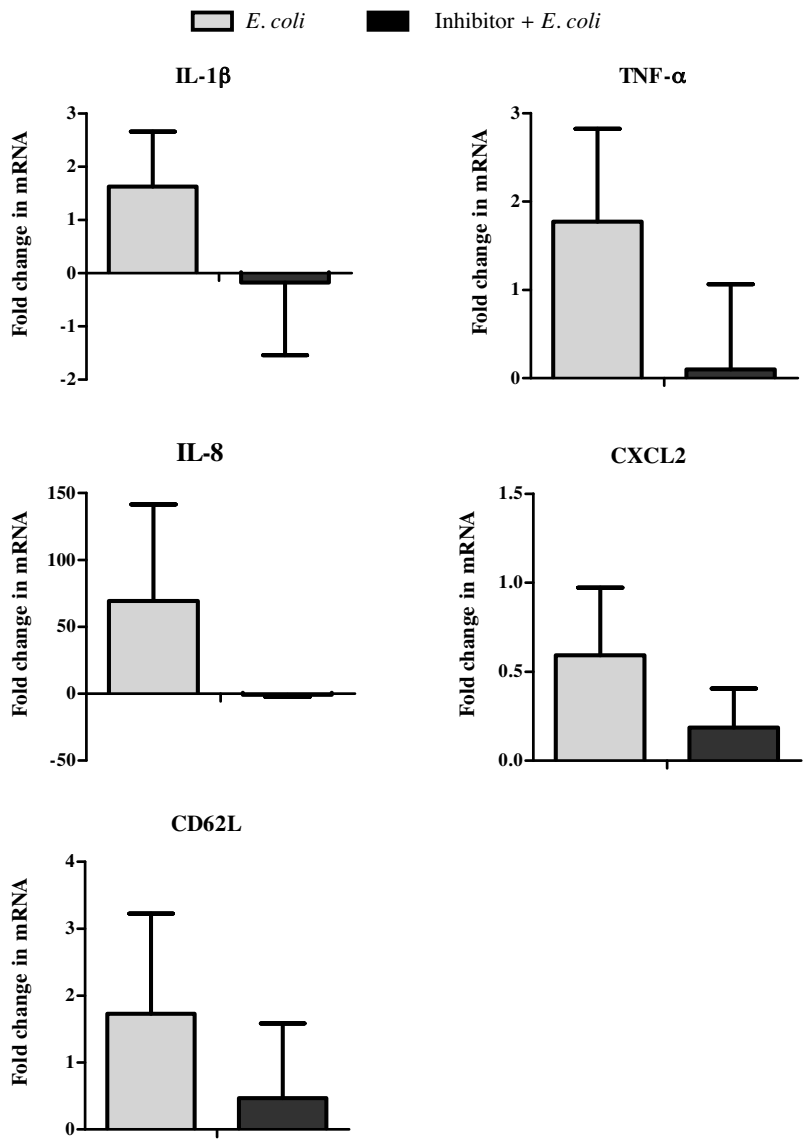

Fig. 2. Inhibition of NOD1-dependent NF-κB pathway on the expression of IL-1β, TNF-α, IL-8, CXCL2 and CD62L in neutrophils exposed to *E. coli*. Quantification of mRNA was performed using SYBR Green-based quantitative real-time PCR (qPCR). Data were analysed using the Pfaffl method. Results (mean ± SEM of 5 heifers) are expressed as fold change over untreated control. Shown is a representative experiment of three independent experiments.

10× TBS pH7.6, 0.1% Tween-20, and 5% w/v of nonfat dry milk), and blotted with the indicated primary antibodies. The primary antibodies used included rabbit polyclonal anti-NF-κB/p65 (1:200, Santa Cruz Biotechnology Inc.) and polyclone mouse anti-β-actin (1:500, Beyotime institute of Biotechnology, Nanjing, China). The sites of antibody binding were visualised by using appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Roche Diagnostic). Band density was measured using ImageJ software (NIH, USA).

2.7. RNA isolation, cDNA synthesis, and qPCR

Total RNA was prepared with TRIzol reagent following the manufacturer's protocol (Takara). The purity of RNA was measured with a spectrophotometer, and the wavelength absorption ratio (260/280 nm) was between 1.6 and 1.8 for all preparations. Isolated RNA (0.5 μg) was reverse-transcribed using the PrimeScript RT reagent Kit with genomic DNA (gDNA) Eraser (Takara). The reaction included one step to eliminate traces of gDNA. Primers including the target and 7 putative candidate reference genes were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast), and are presented in Supplementary Table S1. All except 18S rRNA possess several introns in their sequences, which allowed designing the intron-spanning primers to further minimise the amplification of contaminant gDNA. Amplification efficiencies were determined for all qPCR assays by calculating a 5-point calibration curve (10-fold serial dilution) from pooled cDNA using the equation $E = 10^{[-1/\text{slope}]} - 1$. For all the primer sets, PCR efficiencies ranged between 92% (CXCL2) and 114% (CD62L) (Table S1, Supplementary material).

SYBR green-based qPCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). PCR reactions were performed in 20-μL volumes containing 2 μL of the 1:50 diluted reverse transcriptions, 0.3 μM of each primers and 10 μL SYBR green Master Rox (Roche Diagnostic), and amplified with the standard temperature profile [10 min 95 °C, 40×(15 s 95 °C, 60 s 58–60 °C)]. A negative control using water instead of cDNA was used to exclude contamination. An additional step involving the generation of a melt curve (60–95 °C) was performed to ensure that the correct product was amplified and quantified.

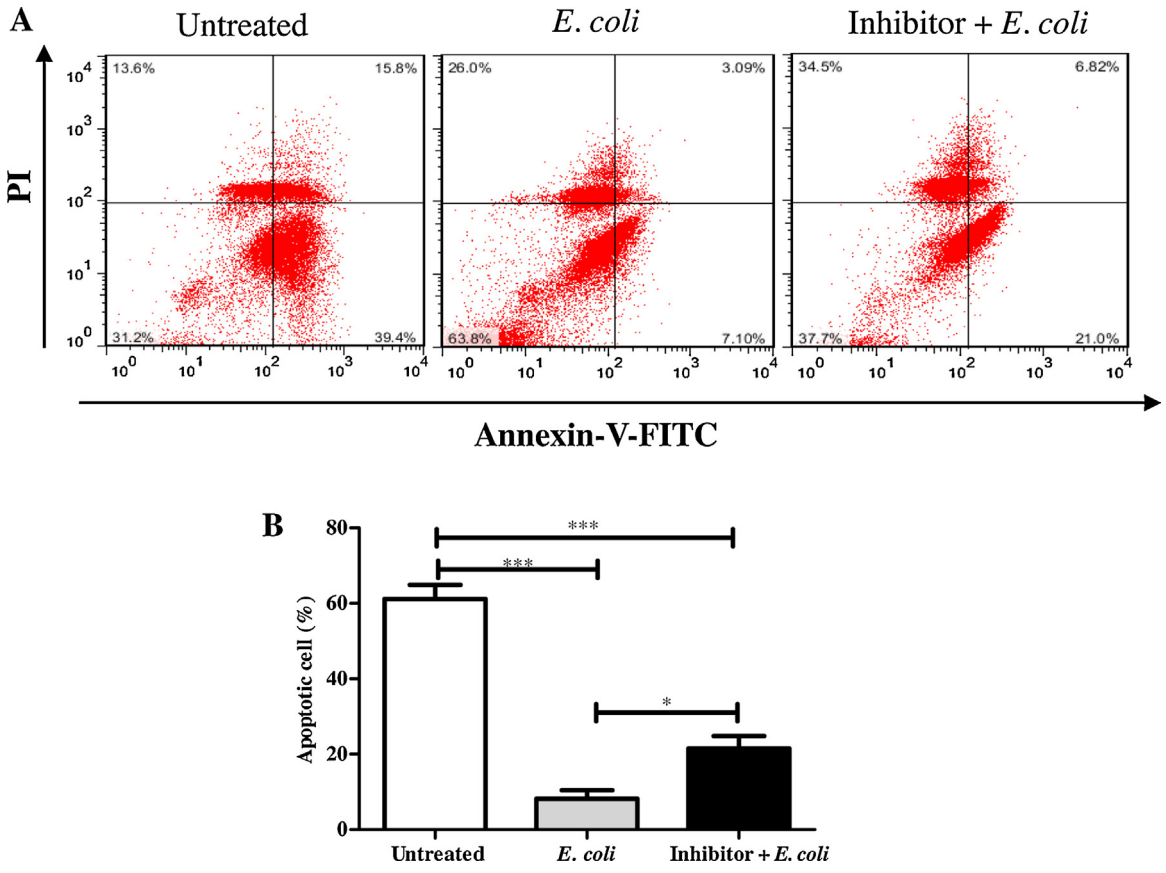

Fig. 3. NOD1/NF-κB pathway contributes to prolonged neutrophil survival upon *E. coli* stimulation. Apoptotic cells were labelled with annexin-V and propidium iodide (PI). (A) Representative FACS plots showing apoptotic cells characterised by annexin-V+/PI− or annexin-V+/PI+. (B) Bar chart corresponds to the percentage of apoptotic cells (mean ± SEM of 6 heifers). * $P < 0.05$ and *** $P < 0.001$.

For normalisation of the qPCR measurements, the stability of 7 putative candidate reference genes (Supplementary Table S1) was assessed. Two reference genes (SDAH and RPL19) were selected based on their lower *M* scores and the pairwise variation $Vn/n+1$ value (Supplementary Fig. S1) as recommended by the geNorm analysis using the Biogazelle qbase + software (Biogazelle NV, Zwijnaarde, Belgium).

Due to the differences in PCR efficiency, the relative expression levels of the target genes were calculated by the Pfaffl analysis method (2001) using the geometric mean of the 2 selected reference genes for normalisation. Final data were expressed as fold change in the expression of the target genes in the treated samples relative to the non-stimulated controls.

2.8. Apoptosis assay

Apoptosis was determined using an Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) kit. Following treatment, 1×10^5 neutrophils were labeled with Annexin V-FITC and PI, according to manufacturer's instructions (Beyotime Institute of Biotechnolgy, Nanjing, China). The samples were analysed using a FACScan flow cytometer (Becton Dickinson).

2.9. Migration assay

Neutrophil migration was assessed using a modified Boyden chamber containing a polycarbonate filter with 5-μm pore size. Neutrophils (2×10^7 cells/well) were pretreated with or without 30 μM ML130 for 2 h, and then 1×10^5 live cells in RPMI medium were placed into the upper chamber. RPMI medium containing *E. coli* (1×10^6 CFU) was loaded into the lower chamber. Following incubation for 1, 2 and 3 h at 37 °C and 5% CO_2, non-migrating cells were completely removed from the top surface of the filters by scraping with a cotton swab. The filters were then gently removed, fixed in 4% paraformaldehyde and stained with haematoxylin. A minimum of 6, 400× fields (0.28 mm^2/field) per filter (33.2 mm^2/filter) were quantified and the mean count per field was used to calculate total number of migrated cells on the whole filter. Cell migration activity was expressed as number of migrated cells/total number of input cells × 100%.

2.10. Detection of phagocytosis and intracellular bacterial killing

Neutrophils were pretreated with ML130 as described above and diluted to 1×10^6 cells/well. Thereafter, cells were incubated with or without *E. coli* (1×10^7 CFU) for additional 30 min. Along with *E. coli* treatment, 100 UI/mL DNase (Roche Diagnostics) was added to each well to inhibit the formation of extracellular neutrophil traps (NET) (Grinberg et al., 2008). Control wells contained bacteria without neutrophils. Samples were centrifuged at 400 × *g* for 3 min, and the supernatant containing bacteria was collected for the determination of extracellular CFU count ($CF_{Uextracellular}$). Neutrophil pellets were treated with 50 μg/mL gentamicin to kill any adherent bacteria and washed twice to remove gentamicin, and lysed with 0.1% Triton on ice for 20 min to allow the release of intracellular bacteria ($CFU_{intracellular}$). All bacterial samples including the control wells ($CF_{Ucontrol}$) were serially diluted and plated onto LB agar to determine the CFU count. Neutrophil phagocytosis was expressed as $(CFU_{control} - CFU_{extracellular})/CFU_{control} \times 100\%$,

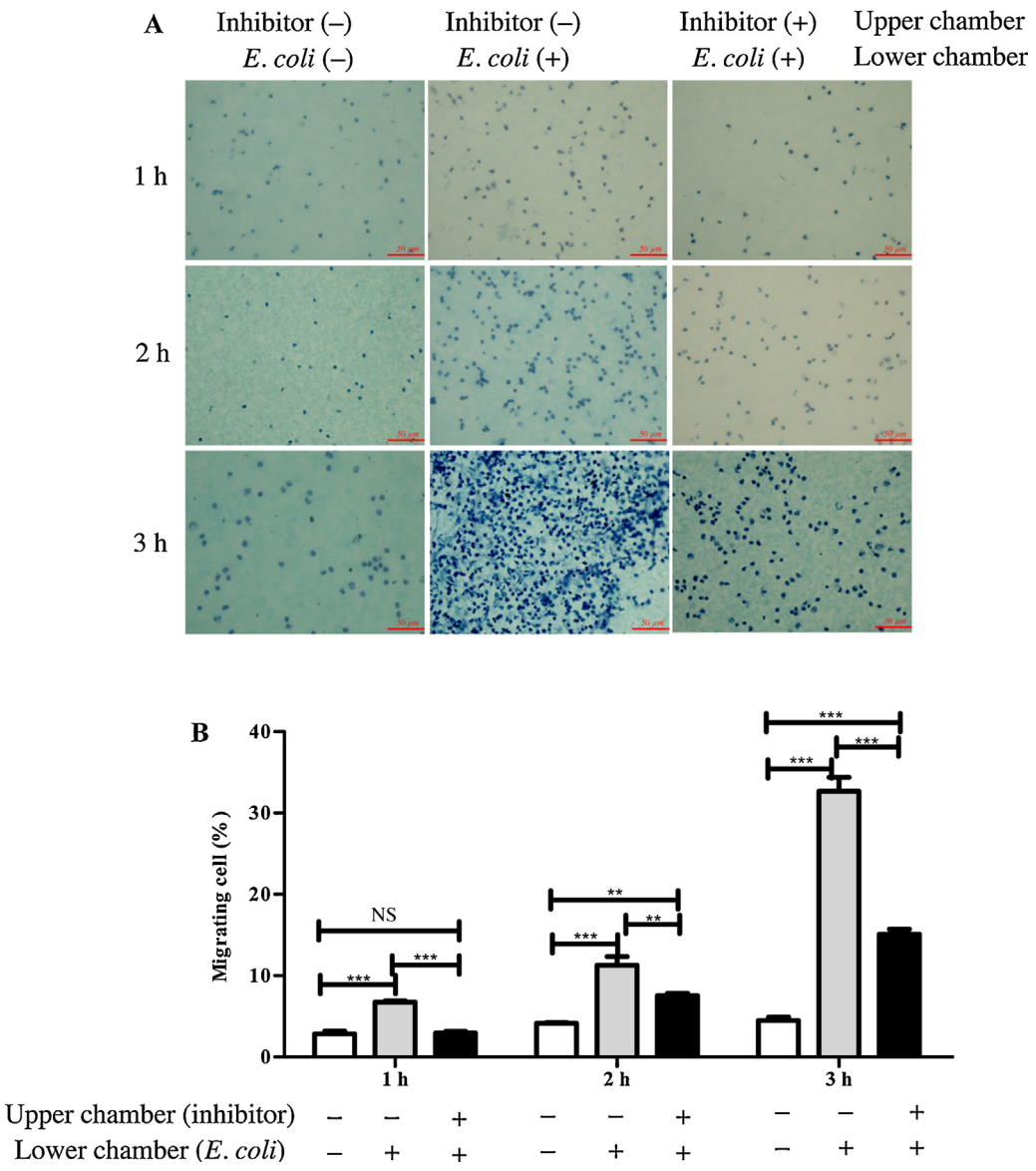

Fig. 4. Involvement of NOD1/NF-κB pathway in *E. coli*-induced neutrophil migration. (A) Representative images showing neutrophils migrated through the Boyden chamber filter toward *E. coli* (scale bar = 50 μm). Neutrophils were loaded into the upper chamber (1×10^5) and allowed to migrate for 1, 2 and 3 h at 37 °C in 5% CO_2 in the presence or absence of *E. coli* (1×10^6 CFU) in the lower chamber. Transmigrated cells were fixed and stained with haematoxylin. (B) A bar graph summarizing the percentage of migrated cell (number of migrated cells/total number of input cells × 100%). The data presented are mean ± SEM of 6 heifers. ** $P < 0.01$ and *** $P < 0.001$. NS: no significant difference.

and intracellular bacterial killing by neutrophils was defined as $(CFU_{control} - CFU_{extracellular} - CFU_{intracellular})/CFU_{control} \times 100\%$.

2.11. Transmission electron microscopy (TEM)

Neutrophil phagocytic activity was further examined by TEM. Following ML130, *E. coli* and gentamicin treatments, neutrophil pellet was fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide. Dehydration was performed in a graded ethanol series and the cells were embedded in Spurr. After polymerisation, specimens were cut at 70–90 nm and contrasted with silver nitrate and lead citrate. Sections were examined and photographed with a Hitachi H-7650TEM.

2.12. Flow cytometric analysis of oxidative burst

Cells were pretreated with ML130 as described above, followed by incubation with dihydrorhodamine 123 (DHR 123, Sigma-Aldrich, Saint Louis, USA) for 5 min. Thereafter, live cells were diluted to 1×10^6 cells/well and incubated in the presence or absence of *E. coli* at a 1:10 cells-to-bacteria ratio for 30 min before flow cytometric analysis (Becton Dickinson). The mean fluorescence intensity (MFI) of rhodamine (ROD) 123 correlating with the mean oxidative burst activity per individual neutrophil (cells undergoing burst, the change from non-fluorescent DHR 123 to fluorescent ROD 123) was recorded, and the percentage of neutrophils having produced reactive oxidants was analysed.

2.13. Statistic analysis

All data were expressed as the mean ± SEM and analysed using Student's *t* test or using one-way ANOVA, with individual group means being compared with the Turkey multiple comparison test (data were checked for normality prior to statistical analyses). Significance was assigned where $P < 0.05$.

3. Results

3.1. ML130 inhibits E. coli-induced nuclear translocation of NF-κB

The effect of ML130 on *E. coli*-induced activation of NF-κB was evaluated by Western blot using the nuclear extracts from neutrophils. As shown in Fig. 1, cells left untreated showed weak NF-κB activity whereas exposure to *E. coli* significantly induced NF-κB nuclear translocation. As expected, treatment with ML130 resulted in a remarkable reduction in *E. coli*-induced NF-κB activation.

3.2. Inhibition of NOD1-dependent NF-κB pathway on interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-8, C-X-C motif ligand 2 (CXCL2) and CD62L expression

We next examined the effect of NOD1-dependent NF-κB pathway impairment on the expression of 5 genes known to play a role in neutrophilic inflammatory/immune processes. The mean fold change in mRNA expression relative to non-stimulated cells is shown in Fig. 2. In this investigation, exposure to *E. coli* for 30 min induced the gene transcription in proinflammatory cytokines IL-1β and TNF-α, chemokine IL-8 and adhesion molecule CD62L, among which the IL-8 exhibited a 68.4-fold relative increase over control cells; however, the expression of chemokine CXCL2 was slightly down-regulated (0.59-fold relative to control level). It is worthy to note that the expression levels between individuals were extremely variable upon *E. coli* stimulation. Treatment with ML130 reduced the relative mRNA levels of all genes investigated in *E. coli*-exposed neutrophils, although there was not a statistically significant difference between the two groups. Still it has to be noted that the levels between individuals were highly variable.

3.3. Effect of NOD1-dependent NF-κB pathway inhibition on neutrophil death upon E. coli infection

Cell death was detected by Annexin V and PI staining and defined as Annexin V-positive. Neutrophils left untreated underwent spontaneous apoptosis by ~60%. Exposure to live *E. coli* significantly promoted cell survival as compared to untreated cells. By comparison, inhibition of NOD1-dependent NF-κB activation caused a significant increase in cell death in *E. coli*-stimulated neutrophils (Fig. 3).

3.4. Inhibition of NOD1-dependent NF-κB pathway on neutrophil migration

We next used a Boyden chamber system to evaluate the effect of NOD1-mediated NF-κB activation on directed migration of neutrophils to *E. coli*. At all time points examined, *E. coli* stimulation significantly induced migration in normal neutrophils. By contrast, the number of cells migrated to *E. coli* was significantly reduced in the presence of ML130 (Fig. 4).

3.5. Inhibition of NOD1/NF-κB pathway on phagocytosis and intracellular bacterial killing by neutrophils

As shown in Fig. 5, ~93% bacteria were phagocytosed by neutrophils in the absence of NOD1/NF-κB inhibitor. However, the ability of phagocytosis was markedly impaired when NOD1-dependent NF-κB activation was inhibited. In accordance with the data of phagocytosis, much less bacteria were killed by neutrophils in the presence of inhibitor ML130. The fraction of ROS-producing neutrophils after stimulation with *E. coli* was reduced in ML130-treated cells compared to their counterparts. Accordingly,

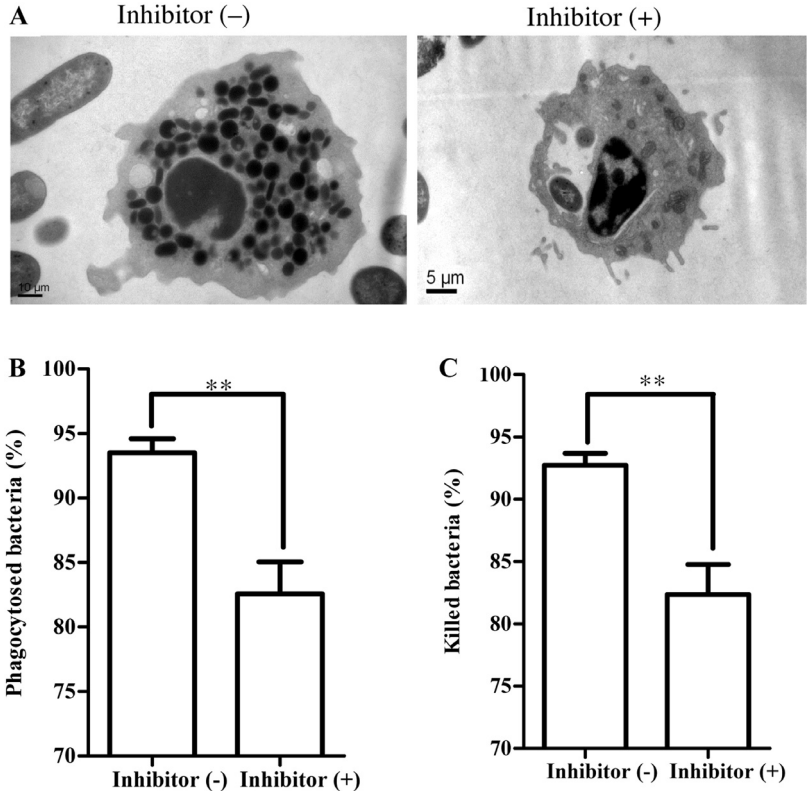

Fig. 5. Effect of NOD1/NF-κB pathway inhibition on bacterial killing in neutrophils. (A) Representative images of *E. coli* phagocytosed by neutrophils. (B) A bar graph summarizing the percentage of bacteria phagocytosed by neutrophils. (C) A bar graph summarizing the percentage of bacteria killed by neutrophils. Data are given as mean ± SEM of 4 heifers. ** $P < 0.01$.

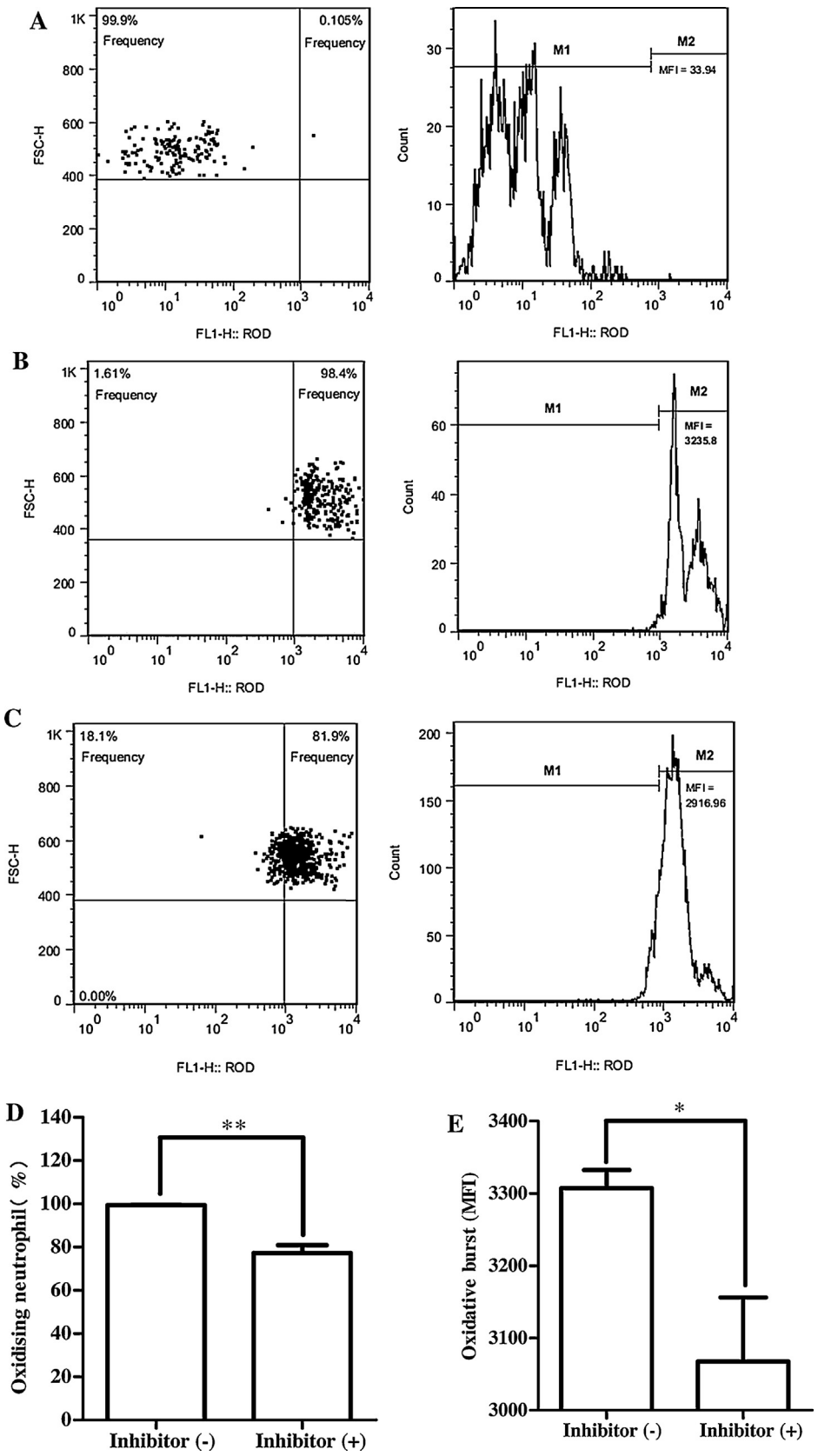

Fig. 6. Inhibition of NOD1/NF-κB pathway impairs oxidative burst by neutrophils. The oxidative burst activity was determined with the probe rhodamine (ROD) 123. Representative fluorescence activated cell sorter (FACS) plots and histograms of neutrophil respiratory burst in (A) resting cells with virtually no oxidative burst [0.106% reactive oxygen species (ROS)-producing cells]; (B) cells stimulated with *E. coli* in the absence of NOD1/NF-κB pathway inhibitor showing an increased fraction of ROS-producing neutrophils (98.4%) and increased ROS production (MFI 3235.8); and (C) cells treated with NOD1/NF-κB pathway inhibitor showing a decreased fraction (81.9%) and ROS release (MFI 2916.96) after incubation with *E. coli*. (D) Data represent the mean ± SEM percentage of neutrophils undergoing oxidative burst ($n = 4$). (E) Data represent the mean fluorescence intensity (MFI) of ROD 123 from neutrophils undergoing oxidative burst ($n = 4$). FSC-H: forward-scatter; FL1-H: fluorescence intensity; M1: non-activated cell population; M2: activated cell population. * $P < 0.05$ and ** $P < 0.01$.

E. coli-induced ROS release, as reflected by the MFI of ROD 123, was significantly inhibited by ML130 (Fig. 6).

4. Discussion

To the best of our knowledge, this is the first study investigating the role of signalling through NOD1 in live *E. coli*-induced early responses of bovine neutrophils. The data reported here demonstrate that NOD1/NF-κB pathway is crucial for cell survival, migration, and phagocytosis and intracellular bacterial killing by bovine neutrophils upon infection. In this context, our study suggests a potential target for regulation of bovine neutrophil function.

In the face of microbial insult, neutrophils are capable of extensive and rapid gene expression changes that are important in the regulation of many neutrophil functions, as well as modulation of the immune response (Hochegger et al., 2007; Newburger et al., 2000; Subrahmanyam et al., 2001; Zhang et al., 2004). NF-κB is a key transcriptional regulator of the genes encoding many proinflammatory cytokines and chemokines (Hayden and Ghosh, 2011). In the present study, exposure to *E. coli* stimulated nuclear translocation of NF-κB in neutrophils, concomitant with increased IL-1β, TNF-α and IL-8 expression; however, the mRNA level of chemokine CXCL2 was slightly reduced, indicating different cytokine responses elicited by *E. coli*. Expression of CD62L, a molecule mediating the initial adhesive interaction of neutrophils to vascular endothelial cells and being constitutively expressed by circulating neutrophils (Weber et al., 2001), was up-regulated as well upon *E. coli* stimulation. This is consistent with a previous study, in which bovine neutrophil demonstrated increased CD62L expression at the early stage of *E. coli*-induced mastitis (Diez-Fraille et al., 2004).

Although neutrophils use a large number of PRRs to sense and respond to various bacterial components with the production of cytokines (Parker et al., 2005; Prince et al., 2011; Sohn et al., 2007), we found that inhibition of NOD1-mediated NF-κB activation had a tendency to suppress the expression of all the cytokines studied in *E. coli*-treated cells, but no statistically significant difference was confirmed. Similarly, transcription of CD62L mRNA in *E. coli*-exposed cells tended to be down-regulated by NOD1 inhibitor. Failure to identify a statistical difference might be attributed to the individual variation of the cows, the small sample size or the relative short-term challenge of *E. coli*. It should be mentioned that changes in mRNA levels do not necessarily reflect parallel changes in the protein products. Further studies are thus warranted to determine whether impairment of NOD1/NF-κB pathway diminishes the protein production of these proinflammatory molecules.

Neutrophils are normally short-lived cells. During the initial phase of inflammation, prolongation of neutrophil lifespan is critical for effective host defense (El and Filep, 2013). Some bacterial components such as LPS, CpG DNA and flagellin were reported to delay apoptotic death in human neutrophils (Francois et al., 2005; Salamone et al., 2010). Likewise, in this work, *E. coli*-stimulated bovine neutrophils demonstrated a significant decrease in cell death. In another study, however, *E. coli*-challenged bovine neutrophils exhibited enhanced apoptosis (Demeyere et al., 2013). The origin of this discrepancy remains unclear and could be related to the differences in design and stimulation. It has been reported that live *E. coli* either inhibit neutrophil apoptosis or induce neutrophil necrosis, depending on bacterial dose (Matsuda et al., 1999). Additionally, in the present study, we found that NOD1/NF-κB pathway contributed significantly to *E. coli*-induced neutrophil survival, indicating that the recognition of *E. coli* by NOD1 plays a crucial role in modulating neutrophil life span. Supporting our results, NOD1 has been shown to prevent apoptosis in other cells (Chen et al., 2008).

It has been reported that dysfunction in NOD1 dramatically reduces the in vitro migration capacity of mouse neutrophils induced by chemoattractant chemokines or formyl-methionyl-leucyl-phenylalanine (fMLP) (Dharancy et al., 2010; Tourneur et al., 2013). In the present study, Boyden chamber assay showed that, upon *E. coli* stimulation in the lower chamber, less neutrophils with impaired intracellular NOD1/NF-κB migrated across the polycarbonate filter as compared to their counterparts left untreated with ML130. Reduced number of migrated neutrophils in ML130-treated cells might be a result of attenuated migration capacity due to inefficiency in NOD1/NF-κB activation, as described by Dharancy et al. (2010). However, as discussed above, inhibition of NOD1/NF-κB pathway led to enhanced neutrophil death in the cell-bacteria coculture system. Although in the Boyden chamber system neutrophils were separated from *E. coli*, an increase of cell death in ML130-treated neutrophils may also occur due to reduced response of those cells to the stimulation of PGN released by *E. coli*. Therefore, we cannot rule out the possible contribution of increased neutrophil death in the reduced migration observed in our in vitro assay. Taken together, data in this work suggest that impairment in NOD1/NF-κB pathway reduces the efficiency of neutrophil migration, although mechanisms underlying this process need further investigations. No previous studies have evaluated the regulatory effect of NOD1 on live *E. coli*-stimulated migratory capacity of neutrophils.

The process of neutrophil bacterial phagocytosis and cell killing comprises a series of transmembrane and intracellular events, starting with the binding and recognition of microbes by specific receptors. Although TLRs have multiple and wide-ranging effects on neutrophils, including oxidative burst and phagocytosis (Hayashi et al., 2003), intracellular NOD1 has emerged as a key factor controlling the ability of neutrophil to engulf and kill pathogens (Clarke et al., 2010). Supporting this work, we found that inhibition of NOD1/NF-κB pathway was sufficient to diminish bacterial uptake and killing by bovine neutrophils, along with reduced ROS production, indicating that signalling through NOD1 is necessary for bacterial killing by bovine neutrophils. The results are in line with our previous study, in which bovine neutrophils responded to NOD1 agonist with a significant increase in phagocytosis and oxidative burst (Tan et al., 2012). Our findings are also supported by Tourneur et al. (2013), who found that mouse blood neutrophils with down-expression in NOD1 exhibited lower capacity to internalise and kill *E. coli*. Others have reported that mouse bone marrow-derived neutrophils deficient in NOD1 are defective in killing pathogens such as Streptococcus pneumoniae and Staphylococcus aureus (Clarke et al., 2010).

5. Conclusion

In summary, this work demonstrates a critical role of NOD1/NF-κB pathway in modulating bovine neutrophil effector responses against live bacteria. Given that immune-compromised neutrophils in mastitis-susceptible periparturient dairy cattle have reduced NOD1 expression (Tan et al., 2012), approaches aiming at restoring neutrophil NOD1 function could be beneficial for prevention or treatment of coliform mastitis.

Conflict of interest statement

None of the author of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.08.010.

References

Burvenich, C., Bannerman, D.D., Lippolis, J.D., Peelman, L., Nonnecke, B.J., Kehrli, M.J., Paape, M.J., 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. J. Dairy Sci. 90 (Suppl. 1), E39–E54.

Burvenich, C., Monfardini, E., Mehrzad, J., Capuco, A.V., Paape, M.J., 2004. Role of neutrophil polymorphonuclear leukocytes during bovine coliform mastitis: physiology or pathology? Verh. K. Acad. Geneeskd. Belg. 66, 150–153, 97–150.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M.A., Foster, S.J., Mak, T.W., Nunez, G., Inohara, N., 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. 4, 702–707.

Chen, G., Shaw, M.H., Kim, Y.G., Nunez, G., 2009. NOD-like receptors: role in innate immunity and inflammatory disease. Annu. Rev. Pathol. 4, 365–398.

Chen, G.Y., Shaw, M.H., Redondo, G., Nunez, G., 2008. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. Cancer Res. 68, 10060–10067.

Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y., Weiser, J.N., 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 16, 228–231.

Clarke, T.B., Weiser, J.N., 2011. Intracellular sensors of extracellular bacteria. Immunol. Rev. 243, 9–25.

Demeyere, K., Remijsen, Q., Demon, D., Breyne, K., Notebaert, S., Boyen, F., Guerin, C.J., Vandenabeele, P., Meyer, E., 2013. *Escherichia coli* induces bovine neutrophil cell death independent from caspase-3/-7/-1, but with phosphatidylserine exposure prior to membrane rupture. Vet. Immunol. Immunopathol. 153, 45–56.

Dharancy, S., Body-Malapel, M., Louvet, A., Berrebi, D., Gantier, E., Gosset, P., Viala, J., Hollebecque, A., Moreno, C., Philpott, D.J., Girardin, S.E., Sansonetti, P.J., Desreumaux, P., Mathurin, P., Dubuquoy, L., 2010. Neutrophil migration during liver injury is under nucleotide-binding oligomerization domain 1 control. Gastroenterology 138, 1546–1556.

Diez-Fraille, A., Mehrzad, J., Meyer, E., Duchateau, L., Burvenich, C., 2004. Comparison of L-selectin and Mac-1 expression on blood and milk neutrophils during experimental *Escherichia coli*-induced mastitis in cows. Am. J. Vet. Res. 65, 1164–1171.

El, K.D., Filep, J.G., 2013. Targeting neutrophil apoptosis for enhancing the resolution of inflammation. Cells 2, 330–348.

Franchi, L., Warner, N., Viani, K., Nunez, G., 2009. Function of Nod-like receptors in microbial recognition and host defense. Immunol. Rev. 227, 106–128.

Francois, S., El, B.J., Dang, P.M., Pedruzzi, E., Gougerot-Pocidalo, M.A., Elbim, C., 2005. Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad. J. Immunol. 174, 3633–3642.

Frutuoso, M.S., Hori, J.I., Pereira, M.S., Junior, D.S., Sonego, F., Kobayashi, K.S., Flavell, R.A., Cunha, F.Q., Zamboni, D.S., 2010. The pattern recognition receptors Nod1 and Nod2 account for neutrophil recruitment to the lungs of mice infected with *Legionella pneumophila*. Microb. Infect. 12, 819–827.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J., Philpott, D.J., 2003a. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 300, 1584–1587.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., Sansonetti, P.J., 2003b. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. 278, 8869–8872.

Grinberg, N., Elazar, S., Rosenshine, I., Shpigel, N.Y., 2008. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*. Infect. Immun. 76, 2802–2807.

Hasegawa, M., Fujimoto, Y., Lucas, P.C., Nakano, H., Fukase, K., Nunez, G., Inohara, N., 2008. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation. EMBO J. 27, 373–383.

Hayashi, F., Means, T.K., Luster, A.D., 2003. Toll-like receptors stimulate human neutrophil function. Blood 102, 2660–2669.

Hayden, M.S., Ghosh, S., 2011. NF-κB in immunobiology. Cell Res. 21, 223–244.

Hochegger, K., Perco, P., Enrich, J., Mayer, B., Mayer, G., Rosenkranz, A.R., Rudnicki, M., 2007. In vitro-transcriptional response of polymorphonuclear leukocytes following contact with different antigens. Eur. J. Clin. Invest. 37, 860–869.

Inohara, N., Koseki, T., Del, P.L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., Nunez, G., 1999. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J. Biol. Chem. 274, 14560–14567.

Kawai, T., Akira, S., 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637–650.

Khan, P.M., Correa, R.G., Divlianska, D.B., Peddibhotla, S., Sessions, E.H., Magnuson, G., Brown, B., Suyama, E., Yuan, H., Mangravita-Novo, A., Vicchiarelli, M., Su, Y., Vasile, S., Smith, L.H., Diaz, P.W., Reed, J.C., Roth, G.P., 2011. Identification of inhibitors of NOD1-induced nuclear factor-kappaB activation. ACS Med. Chem. Lett. 2, 780–785.

Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. 11, 519–531.

Masumoto, J., Yang, K., Varambally, S., Hasegawa, M., Tomlins, S.A., Qiu, S., Fujimoto, Y., Kawasaki, A., Foster, S.J., Horie, Y., Mak, T.W., Nunez, G., Chinnaiyan, A.M., Fukase, K., Inohara, N., 2006. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. J. Exp. Med. 203, 203–213.

Matsuda, T., Saito, H., Inoue, T., Fukatsu, K., Lin, M.T., Han, I., Furukawa, S., Ikeda, S., Muto, T., 1999. Ratio of bacteria to polymorphonuclear neutrophils (PMNs) determines PMN fate. Shock 12, 365–372.

Moreira, L.O., Zamboni, D.S., 2012. NOD1 and NOD2 signaling in infection and inflammation. Front. Immunol. 3, 328.

Newburger, P.E., Subrahmanyam, Y.V., Weissman, S.M., 2000. Global analysis of neutrophil gene expression. Curr. Opin. Hematol. 7, 16–20.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C., 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J. Mammary Gland Biol. Neoplasia 7, 109–121.

Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: structure and function in blood and milk. Vet. Res. 34, 597–627.

Parker, L.C., Whyte, M.K., Dower, S.K., Sabroe, I., 2005. The expression and roles of Toll-like receptors in the biology of the human neutrophil. J. Leukoc Biol. 77, 886–892.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Prince, L.R., Whyte, M.K., Sabroe, I., Parker, L.C., 2011. The role of TLRs in neutrophil activation. Curr. Opin. Pharmacol. 11, 397–403.

Ratner, A.J., Aguilar, J.L., Shchepetov, M., Lysenko, E.S., Weiser, J.N., 2007. Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. Cell Microbiol. 9, 1343–1351.

Salamone, G.V., Petracca, Y., Fuxman, B.J., Rumbo, M., Nahmod, K.A., Gabelloni, M.L., Vermeulen, M.E., Matteo, M.J., Geffner, J.R., Trevani, A.S., 2010. Flagellin delays spontaneous human neutrophil apoptosis. Lab Invest. 90, 1049–1059.

Segal, A.W., 2005. How neutrophils kill microbes. Annu. Rev. Immunol. 23, 197–223.

Sohn, E.J., Paape, M.J., Connor, E.E., Bannerman, D.D., Fetterer, R.H., Peters, R.R., 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38, 809–818.

Stevens, M.G., De Spiegeleer, B., Peelman, L., Boulougouris, X.J., Capuco, A.V., Burvenich, C., 2012. Compromised neutrophil function and bovine *E. coli* mastitis: is C5a the missing link? Vet. Immunol. Immunopathol. 149, 151–156.

Subrahmanyam, Y.V., Yamaga, S., Prashar, Y., Lee, H.H., Hoe, N.P., Kluger, Y., Gerstein, M., Goguen, J.D., Newburger, P.E., Weissman, S.M., 2001. RNA expression patterns change dramatically in human neutrophils exposed to bacteria. Blood 97, 2457–2468.

Tamassia, N., Le Moigne, V., Calzetti, F., Donini, M., Gasperini, S., Ear, T., Cloutier, A., Martinez, F.O., Fabbri, M., Locati, M., Mantovani, A., McDonald, P.P., Cassatella, M.A., 2007. The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. J. Immunol. 178, 7344–7356.

Tan, X., Li, W.W., Guo, J., Zhou, J.Y., 2012. Down-regulation of NOD1 in neutrophils of periparturient dairy cows. Vet. Immunol. Immunopathol. 150, 133–139.

Thomas, C.J., Schroder, K., 2013. Pattern recognition receptor function in neutrophils. Trends Immunol. 34, 317–328.

Tourneur, E., Ben, M.S., Chassin, C., Bens, M., Goujon, J.M., Charles, N., Pellefigues, C., Aloulou, M., Hertig, A., Monteiro, R.C., Girardin, S.E., Philpott, D.J., Rondeau, E., Elbim, C., Werts, C., Vandewalle, A., 2013. Cyclosporine A impairs nucleotide binding oligomerization domain (Nod1)-mediated innate antibacterial renal defenses in mice and human transplant recipients. PLOS Pathog. 9, e1003152.

Weber, P.S., Madsen, S.A., Smith, G.W., Ireland, J.J., Burton, J.L., 2001. Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet. Immunol. Immunopathol. 83, 213–240.

Zhang, X., Kluger, Y., Nakayama, Y., Poddar, R., Whitney, C., DeTora, A., Weissman, S.M., Newburger, P.E., 2004. Gene expression in mature neutrophils: early responses to inflammatory stimuli. J. Leukoc Biol. 75, 358–372.