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Role of the NOD1/NF-κB pathway on bovine neutrophil responses to crude lipopolysaccharide

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Highlights

- *Inhibition of NOD1/NF-κB by ML130 decreases IκBα phosphorylation in crude lipopolysaccharide (cLPS)-stimulated bovine neutrophils.*
- Blocking the *NOD1/NF-κB pathway* inhibits bovine *neutrophil migration* and phagocytic killing capacity upon *cLPS stimulation.*
- *NOD1/NF-κB pathway* inhibition *promotes neutrophil death in response to cLPS stimulation.*
- *I*nhibition of the NOD1/NF-κB pathway depresses the functional responses of bovine neutrophils to cLPS.
- *Reduced neutrophil NOD1 expression during the periparturient period might play a role in the pathogenesis of coliform mastitis in cattle*.

Abstract

Cytosolic nucleotide oligomerisation domain (NOD)-like receptors play an important role in host defence against infection. Reduced NOD1 expression has been observed in dysfunctional neutrophils derived from periparturient cattle known to be most susceptible to coliform mastitis. However, whether impairment of NOD1 suppresses the immune responses of bovine neutrophils during bacterial infections remains unknown. Crude (phenol extracted) lipopolysaccharide (cLPS), which often contains other immunostimulatory molecules, including NOD1 agonist, is known to induce almost the whole bacterial response. This study was conducted to explore the role of NOD1/nuclear factor (NF)-κB pathway in the cytokine and functional responses of bovine neutrophils challenged with *Escherichia coli*-derived cLPS. Freshly isolated blood neutrophils from healthy heifers were pre-incubated for 2 h with ML130, a selective inhibitor of NOD1/NF-κB pathway. Cells were then exposed to cLPS for additional 4 h. Inhibition of the NOD1/NF-κB

pathway resulted in a decrease in cLPS-induced phosphorylation of the inhibitor of NF-κBα (IκBα) in neutrophils. Impairment of the NOD1/NF-κB pathway also down-regulated mRNA levels of pro-inflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, chemokines IL-8 and C-X-C motif ligand 2 (CXCL2), and adhesion molecules CD11b and CD62L, in cLPS-challenged cells. Functional analyses showed that blocking the NOD1/NF-κB pathway inhibited neutrophil migration and phagocytic killing capacity, and promoted neutrophil death upon cLPS stimulation. The data presented here demonstrate that activation of NOD1/NF-κB pathway contributes to the functional responses of neutrophils to cLPS.

Keywords: Bovine; Cytosolic nucleotide oligomerisation domain 1; Immune response; Neutrophils

Introduction

Mastitis is a common and costly disease affecting dairy cattle worldwide. Cows are most susceptible to intramammary infections caused by environmental bacteria, particularly *Escherichia coli*, shortly after calving and during early lactation (Stevens et al., 2012). In the mammary gland, an effective defence against invading pathogens depends on the rapid influx of neutrophils from the circulation and subsequent phagocytosis and killing of bacteria (Paape et al., 2002). Neutrophil dysfunction may contribute to the increased incidence and severity of coliform mastitis during periparturient period, although the mechanisms underlying neutrophil dysfunction have not been elucidated (Burvenich et al., 2003; Diez-Fraile et al., 2003a; Stevens et al., 2012; Zoldan et al., 2014).

The initial host defence against bacterial infections is executed essentially by a number of pattern recognition receptors (PRRs) involving the membrane-associated Toll-like receptors (TLRs) and cytosolic nucleotide oligomerisation domain (NOD)-like receptors (NLRs) (Mogensen, 2009). The two best-characterised members of the NLR family are NOD1 and NOD2, which recognise distinct sub-structures from the synthesis and/or degradation of bacterial peptidoglycan (PGN). While NOD1 senses γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) derived primarily from Gram negative bacilli (Chamaillard et al., 2003), NOD2 is activated by muramyl dipeptide (MDP), a conserved structure common to all bacteria (Girardin et al., 2003). Similar to TLRs, activation of NODs initiates an intracellular cascade of events culminating in nuclear factor (NF)-κB activation via the phosphorylation of inhibitor of NF-κBα (IκBα) (Kawai and Akira, 2009). Although NODs act independently of TLRs, there is evidence that NODs are essential for efficient bacterial clearance and mouse survival when TLR signalling is compromised (Kim et al., 2008). More

specifically, NOD1 has been shown to be necessary for the phagocytic bacterial killing by mouse neutrophils (Clarke et al., 2010).

Lipopolysaccharide (LPS) is an abundant glycolipid in the outer membrane of Gram negative bacteria and can induce powerful inflammatory responses through the TLR4 complex during bacterial infection. Impairment of the neutrophil TLR4 pathway may be involved in the pathogenesis of periparturient *E. coli* mastitis (De Schepper et al., 2008). However, no conclusive data are available for supporting this assumption, although decreased expression of some genes downstream of TLR4 in neutrophils derived from early lactating cows has been observed (Stevens et al., 2011). In addition to TLR4, bovine neutrophils express NOD1 and NOD2 (Worku and Morris, 2009; Tan et al., 2012).

Recently, we found that the expression of NOD1, but not NOD2, in blood neutrophils of periparturient cows was markedly reduced, resulting in diminished bacterial killing activity upon NOD1 agonist stimulation (Tan et al., 2012). However, as in infection, multiple PRRs may be simultaneously activated, but whether a down-regulation of NOD1 is sufficient to suppress the responses of bovine neutrophils to infection remains unknown.

Crude (phenol extract) LPS (cLPS) is able to mimic whole bacteria and accounts for almost the entire bacterial response (Huang et al., 2001); cLPS is commonly used in vivo and in vitro to study the host innate immune response during coliform mastitis (Klesius et al., 1984; Sohn et al., 2007a, b; Revelo and Waldron, 2012). Notably, cLPS often contains other immunostimulatory molecules, such as nucleic acids, capsular polysaccharides and PGN fragments (Tirsoaga et al.,

2007), and can induce NOD1-dependent NF-κB activation (Inohara et al., 2001; Chamaillard et al., 2003). Thus, cLPS-activated neutrophils could provide a good model to investigate the contribution of NOD1 in the responses of neutrophils to whole *E. coli*. The present study was conducted to investigate the effect of NOD1/NF-κB inhibition on cytokine responses, migration, phagocytic killing capacity and survival of neutrophils challenged by *E. coli*-derived cLPS.

Materials and methods

Blood collection

This study was carried out using peripheral blood samples from Chinese Holstein heifers aged 8-9 months. Heifers were fed grass and corn silage and hay. Peripheral blood was collected from the tail veins into plastic tubes containing 10% by volume of acid citrate dextrose (ACD) anticoagulant. All heifers appeared to be clinically healthy on the day of sampling. The study was approved by the Ethical Committee for Animal Welfare of Zhejiang University (approval number 120359; date of approval 10 November 2012)

Preparation of cells

Neutrophils were isolated as previously described (Tan et al., 2012). Whole blood was centrifuged at 1000 *g* for 20 min and the plasma, buffy coat and upper layer of packed red blood cells were removed. After hypotonic lysis of erythrocytes, the sample was centrifuged and the cell pellet was washed twice in cold phosphate buffered saline (PBS, pH 7.4). Viability of isolated neutrophils, as determined by trypan blue exclusion, was never <95%. Cells were suspended in RPMI 1640 containing 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell treatment

Neutrophils were placed onto 24-well plates at 2 x 10^7/well. The cells were incubated with or without 30 μmol/L ML130 (Selleckchem), a potent and selective inhibitor of NOD1-induced NF-κB activation (Khan et al., 2011), for 2 h at 37 °C, and then exposed to 100 ng/mL cLPS (*E. coli* serotype 0111:B4, phenol extract, Sigma-Aldrich) (Sohn et al., 2007b) or 10 μg/mL NOD1 agonist C12-iE-DAP (InvivoGen) (Tan et al., 2012), or were left non-stimulated at 37 °C for a further 4 h. In our preliminary studies, we treated neutrophils with 10, 30 or 90 μmol/L ML130 for 2 h and we determined that this molecule alone did not have a significant effect on the phosphorylation of inhibitor of NF-κBα (IκBα) or neutrophil phagocytosis (data not shown); therefore, we used 30 μmol/L ML130 to inhibit NOD1/NF-κB pathway in activated neutrophils.

Western blot analysis

Protein expression was detected in whole cell lysates. Cells were lysed using ice cold radioimmunoprecipitaion assay (RIPA) buffer. The total protein concentration of the lysates was determined using the bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology). Samples were separated at equal protein concentrations (50 μg) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred to nitrocellulose membranes. Membranes were blocked with bovine serum albumin (BSA) and incubated at 4 °C overnight with a polyclonal antibody against phosphor (p)-IκBα (1:1000, Ser 32/36, Santa Cruz Biotech), NOD1 (1:500, E-14, Santa Cruz Biotech) or a monoclonal antibody against β-actin (1:500, AC-74, Byotime Biotechnology, China). The primary antibody was detected using an appropriate horseradish peroxidase-conjugated secondary antibody (Byotime

Biotechnology, China) and signals were visualised using an electrochemiluminescent (ECL) detection system (Roche Diagnostic). Densitometry was performed using Quantity One software (Bio-Rad Laboratories).

RNA preparation, cDNA synthesis, reference gene selection and quantitative real-time PCR analysis

Total RNA extraction was performed by adding 1 mL of TRIzol (Takara) to each well. The cDNA was reverse transcribed with 0.5 µg RNA using the PrimeScript RT reagent Kit with genomic DNA (gDNA) Eraser (Takara), which includes a gDNA elimination pre-treatment of RNA sample. Intron-spanning oligonucleotide primers for quantitative real-time PCR (qPCR) were designed using the Primer-BLAST programme[1] (see Appendix: Supplementary Table S1).

Four reference genes (*ACTIN*, *18S rRNA*, *RLP19* and *YWHAZ*) for normalisation of qPCR measurements were selected from seven candidate genes on the basis of their stable expression profiles across treatments, as recommended by the geNorm analysis using the Biogazelle qbase+ software (Biogazelle NV) (see Appendix: Supplementary Fig. S1). Amplification efficiencies were determined for all qPCR assays by calculating a five-point calibration curve (10-fold serial dilution) from pooled cDNA using the equation $E = 10^{[-1/slope]}-1$. For all the primer sets, PCR efficiencies ranged between 92 (CXCL2) and 114% (CD62L) (see Appendix: Supplementary Table S1).

The resulting cDNA (2 µL, 1:20 diluted) was applied to qPCR analyses (20 µL final volume) with 0.3 µmol/L gene-specific primers (see Appendix: Supplementary Table S1) in 10 µL SYBR

[1] See: http://www.ncbi.nlm.nih.gov/tools/primer-blast.

green Master Rox (Roche Diagnostic) and amplified with the standard temperature profile (10 min at 95 °C, 40 cycles at 15 s for 95 °C, then 45 s at 58-60 °C) in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). A negative control using pure 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. The relative expression of target genes was calculated by the Pfaffl method (2001) using the geometric mean of the cycle threshold (Ct) values of the four selected reference genes for normalisation. Data reported are the fold change in the expression of the target genes in the treated samples relative to the controls.

Migration assay

Cell migratory ability was assessed using the 24-well Transwell plate system (Corning Costa). The lower well was separated from the upper well by a polycarbonate filter with an 8 µm pore diameter. Neutrophils (3×10^4) in 150 µL serum-free medium were placed into the upper well, then 0.5 mL of RPMI 1640 containing 10% FBS was added into the lower well. After incubation for 4 h at 37 °C, the cells that had migrated through the filter into the lower well were counted using a haemocytometer. The experiments were performed in triplicate.

Detection of apoptosis

Apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Beyotime Institute of Biotechnology), which distinguishes apoptotic cells (Annexin V-FITC positive, PI negative) from necrotic cells (Annexin V-FITC positive, PI positive). Following treatments, cells were collected and washed with PBS, then 1×10^5

cells were incubated with 200 μL Annexin V-FITC buffer for 20 min at room temperature, followed by 10 μL of PI solution for another 10 min. The samples were subsequently analysed using a FACScan flow cytometer (Becton Dickinson).

Determination of phagocytosis and phagocytosis-dependent oxidative burst

Fluorescent microspheres (φ=1.81 μm, Spherotech) were used to measure phagocytosis, while dihydrorhodamine 123 (DHR123, Sigma-Aldrich) was used to quantify oxidative burst (Tan et al., 2012). ACD anticoagulated blood (100 μL) was pre-treated with or without ML130 (30 μmol/L) for 2 h at 37 °C. After incubation in the presence or absence of 100 ng/mL cLPS for another 2 h, DHR 123 was introduced into the samples and the mixture was incubated for 15 min at 37 °C. Samples were then incubated with opsonised fluorescent microspheres for 30 min at 37 °C. The phagocytic activity and reactive oxygen species (ROS) generation was monitored on a FACScan flow cytometer (Beckman Coulter). Histograms were used to plot the percentage of fluorescence positive cells and mean fluorescence intensity (MFI, correlated with the mean number of beads ingested by single phagocytes), and the mean oxidative burst activity of single phagocytes (change from dihydrorhodamine 123 to rhodamine 123). An index of overall phagocytic or oxidative burst activity was calculated by multiplying the percentage of responding cells by the corresponding MFI: Index = (% positive cells) x (log MFI)/100 (Tan et al., 2012).

Statistical analysis

Data are reported as means ± standard deviations (SDs). Differences in gene expression were compared using the non-parametric Mann-Whitney *U* test. The statistical significance of cell apoptosis was determined using Kruskal-Wallis test, since the data were not normally distributed.

Other data were analysed using one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. The software used was SPSS 16.0 for Windows. Differences were considered to be significant at $P < 0.05$.

Results

To determine whether ML130 inhibits NOD1-activated NF-κB pathway in neutrophils, we treated neutrophils with ML130 for 2 h prior to addition of the NOD1 agonist iE-DAP. iE-DAP induced a significant increase in IκBα phosphorylation, which was inhibited by ML130. cLPS stimulated IκBα phosphorylation as well (P <0.001 vs. basal). However, the cLPS-induced phosphorylation of IκBα was significantly blocked by ML130 (Fig. 1).

The effect of ML130 on NOD1expression in cLPS-stimulated neutrophils was also determined. Exposure of bovine neutrophils to cLPS resulted in a significant elevation of NOD1 protein ($P < 0.05$ vs. basal). ML130 showed no effect on NOD1 expression under basal conditions ($P = 0.098$; Fig. 2).

cLPS stimulation resulted in an increase in mRNA levels of interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-8, C-X-C motif ligand 2 (CXCL2), CD62L and CD11b relative to the untreated controls, although mRNA levels between individuals were highly variable. ML130 treatment had no significant effect on these mRNA levels in cLPS-challenged cells (Fig. 3).

We further investigated the role of the NOD1/pathway in the migration of cLPS-challenged neutrophils. As shown in Fig. 4, cLPS stimulation enhanced neutrophil migration relative to the

225 basal level. However, this migration-inducing effect of cLPS was significantly inhibited by ML130.

226

227 Apoptosis of neutrophils treated with cLPS in the presence or absence of NOD1/NF-κB
228 inhibitor is shown in Fig. 5. Exposure to cLPS caused a moderate decrease in neutrophil death (*P*
229 =0.052). However, inhibition of NOD1 signalling significantly promoted neutrophil apoptosis upon
230 cLPS stimulation.

231

232 Bovine neutrophils displayed enhanced phagocytic capacity and phagocytosis-dependent
233 ROS generation following cLPS stimulation (Fig. 6). cLPS-challenged cells exhibited attenuated
234 phagocytosis as well as oxidative burst when NOD1/NF-κB pathway was inhibited.

235

236 **Discussion**

237 We previously demonstrated that dysfunctional neutrophils derived from periparturient dairy
238 cows had reduced NOD1 expression (Tan et al., 2012). In the present study, we investigated
239 whether impairment of NOD1/NF-κB is sufficient to influence the responses of bovine neutrophils
240 to cLPS. Inhibition of the NOD1/NF-κB pathway attenuated cLPS-induced cell survival, migration,
241 phagocytic bacterial killing and, to some extent, the gene transcription of pro-inflammatory
242 mediators.

243

244 ML130 is a potent and selective inhibitor of NOD1-dependent NF-κB activation (Khan et al.,
245 2011). In the present study, we first verified the inhibitory effect of ML130 on the NOD1/NF-κB
246 pathway by using cells exposed to the NOD1 agonist iE-DAP. The presence of NOD1 agonists in
247 cLPS were then confirmed in that cLPS-induced NF-κB activation, as reflected by increased IκBα

phosphorylation, was significantly inhibited by ML130. We have tested the effects of several doses of iE-DAP on neutrophil activation in terms of IκBα phosphorylation and phagocytic function and it appears that high doses of this molecule are required to activate neutrophils (data not shown). The mechanisms by which neutrophils become sensitive to low amounts of NOD1 agonists in cLPS need to be further investigated. However, a synergistic interplay between TLR4 and NOD1 might contribute to this process. Synergistic interactions between TLRs and NODs in the induction of innate immune responses have been reported (Fritz et al., 2005; van Heel et al., 2005).

cLPS-challenged neutrophils also had increased NOD1 expression, suggesting that up-regulation of NOD1 may be part of an effective innate response of neutrophils against bacterial infections. Previous studies have demonstrated a key role of NF-κB in controlling NOD gene expression in other cells (Takahashi et al., 2006; Muhlbauer et al., 2008). Consistent with these findings, we found that inhibition of NOD1-dependent NF-κB activation led to a modest decrease in NOD1 expression in cLPS-challenged neutrophils, indicating that impairment of the NOD1/NF-κB pathway might limit the expression of neutrophil NOD1 during infection.

Activated neutrophils are able to synthesise a broad range of pro-inflammatory mediators through the NF-κB pathway, thereby regulating both innate and acquired immunity (Cloutier et al., 2007). In agreement with some previous studies (Xing and Remick, 2003; Sohn et al., 2007b), bovine neutrophils stimulated by cLPS showed enhanced NF-κB activation, concomitant with up-regulated IL-1β, TNF-α, IL-8 and CXCL2 mRNA expression. In addition, the expression of CD62L and CD11b, which are involved in neutrophil diapedesis by mediating the adherence of circulating neutrophils to microvascular endothelium (Diez-Fraile et al, 2003b; 2004), was

up-regulated by cLPS as well.

Recent literature suggests that NOD1-dependent activation of NF-κB contributes significantly to cLPS-induced production of pro-inflammatory cytokines in cells possessing TLR4 (Zheng et al., 2012). There is also evidence that NOD1 signalling regulates CD11b expression on mouse neutrophils (Dharancy et al., 2010). Induction of certain proteins in neutrophils is often preceded by an increased accumulation of the related mRNA transcripts (Tecchio et al., 2014). Further studies are needed to determine whether impairment of the NOD1/NF-κB pathway is sufficient to diminish the cLPS-induced protein production of these pro-inflammatory molecules.

Once neutrophils have left the circulation, they migrate towards infected tissue through chemotaxis. Neutrophils from NOD1-defective mice have reduced chemotactic migration capacity (Clarke et al., 2010; Dharancy et al., 2010). In the present study, we found that inhibition of the NOD1-dependent NF-κB pathway was sufficient to inhibit cLPS-stimulated neutrophil chemotactic migration (we used serum as chemoattractant), even though TLR4 ligands constitute a major component of unpurified LPS. Activation of TLRs, including TLR2 and TLR4, leads to reduced chemotaxis by human neutrophils (Hayashi et al., 2003). Aomatsu et al. (2008) provided further evidence that a TLR4 agonist induces a random rather than chemotactic migration of human neutrophils. Tourneur et al. (2013) demonstrated that NOD1 plays a critical role for neutrophils to migrate into tissues infected with *E. coli*. Given that impaired neutrophil chemotaxis is involved in the pathogenesis of periparturient mastitis (Cai et al., 1994), our results allow us to argue that reduced neutrophil NOD1 expression during the periparturient period might predispose cows to coliform mastitis.

LPS delays neutrophil apoptosis by activation of various pathways, including NF-κB (Francois et al., 2005; Dick et al., 2009). In contrast, NOD1 activation either induces or inhibits apoptosis, depending on cell types (Chen et al., 2008; Shigeoka et al., 2010; Fernandez-Velasco et al., 2012). Accelerated apoptosis of neutrophils has been reported in postpartum cows with naturally occurring acute coliform mastitis (Tharwat, 2011). Supporting a previous study with human neutrophils (Sabroe et al., 2003), we found that cLPS induced a modest decrease of apoptosis in bovine neutrophils. Conversely, inhibition of NOD1-mediated NF-κB activation caused a significant increase in cell death in cLPS-challenged cells. Extending the lifespan of neutrophils at the site of infection is critical for efficient elimination of invading pathogens (Nathan, 2006; Savill et al., 2002). Therefore, impairment of the NOD1/NF-κB pathway might partly account for the increased susceptibility of periparturient cows to coliform mastitis by reducing neutrophil survival in the early stages of infection.

Reduced neutrophil phagocytosis and impaired oxidative burst have been implicated in the pathogenesis of periparturient mastitis (Vangroenweghe et al., 2005). We have previously reported that NOD1 activation induces phagocytosis and enhanced oxidative burst in bovine neutrophils (Tan et al., 2012). It has also been documented that stimulation of individual TLRs on human neutrophils results in an increased phagocytic response (Hayashi et al., 2003). In the present study, impairment of the NOD1/NF-κB pathway led to a significant reduction in the phagocytic activity of cLPS-challenged neutrophils, accompanied by reduced phagocytosis-associated ROS generation. These results indicate that NOD1-dependent NF-κB activation may be required for bovine neutrophils to engulf and kill *E. coli*. Mouse neutrophils with depressed NOD1 expression had

317 significantly lower ex vivo capacity to phagocytise and kill *E. coli* (Tourneur et al., 2013). NOD1$^{-/-}$
318 neutrophils form mice had a lower capacity for bacterial phagocytic killing than wild-type
319 neutrophils (Clark et al., 2010; Dharancy et al., 2010).
320
321 Primary neutrophils are terminally differentiated and short lived cells and thus are not
322 amenable to genetic manipulation. We therefore used an inhibitor ML130 rather than gene silencing
323 to study the function of NOD1. Under this circumstance, undesirable off-target effects produced by
324 the inhibitor used might not be excluded. Moreover, activation of other inflammatory pathways
325 downstream of NOD1, for example, the mitogen-activated protein kinase (MAPK) pathway
326 (Strober et al., 2006), might counteract the effects generated by NOD1/NF-κB pathway impairment.
327 For a better understanding of the role of NOD1 in cLPS-induced neutrophil responses, further
328 studies should be carried out using in vitro-derived neutrophils that are capable of gene
329 modification (McDonald et al., 2011). Since cLPS cannot fully represent the *E. coli* bacteria
330 themselves in terms of the proportion of NOD1 to TLR4 agonists, further studies are warranted to
331 determine whether NOD1 signalling impairment suppresses the immune responses of neutrophils to
332 live *E. coli*.
333
334 **Conclusions**
335 This study demonstrates that inhibition of the NOD1/NF-κB pathway depresses the
336 functional responses of neutrophils to cLPS. The results raise the possibility that reduced neutrophil
337 NOD1 expression may be involved in the pathogenesis of coliform mastitis in periparturient dairy
338 cows.
339

Conflict of interest statement

None of the authors 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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References

Aomatsu, K., Kato, T., Fujita, H., Hato, F., Oshitani, N., Kamata, N., Tamura, T., Arakawa, T., Kitagawa, S., 2008. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology 123, 171-180.

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L., 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Veterinary Research 34, 521-564.

Cai, T.Q., Weston, P.G., Lund, L.A., Brodie, B., McKenna, D.J., Wagner, W.C., 1994. Association between neutrophil functions and periparturient disorders in cows. American Journal of Veterinary Research 55, 934-943.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., et al., 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nature Immunology 4, 702-707.

Chen, G.Y., Shaw, M.H., Redondo, G., Nunez, G., 2008. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. Cancer Research 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. Nature Medicine 16, 228-231.

Cloutier, A., Ear, T., Blais-Charron, E., Dubois, C.M., McDonald, P.P., 2007. Differential involvement of NF-κB and MAP kinase pathways in the generation of inflammatory cytokines

by human neutrophils. Journal of Leukocyte Biology 81, 567-577.

De Schepper, S., De Ketelaere, A., Bannerman, D.D., Paape, M.J., Peelman, L., Burvenich, C., 2008. The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of *Escherichia coli* mastitis in dairy cattle. Veterinary Research 39, 5.

Dharancy, S., Body-Malapel, M., Louvet, A., Berrebi, D., Gantier, E., Gosset, P., Viala, J., Hollebecque, A., Moreno, C., Philpott, D.J., et al., 2010. Neutrophil migration during liver injury is under nucleotide-binding oligomerization domain 1 control. Gastroenterology 138, 1546-1556, 1556.e1-5.

Dick, E.P., Prince, L.R., Prestwich, E.C., Renshaw, S.A., Whyte, M.K., Sabroe, I., 2009. Pathways regulating lipopolysaccharide-induced neutrophil survival revealed by lentiviral transduction of primary human neutrophils. Immunology 127, 249-255.

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. American Journal of Veterinary Research 65, 1164-1171.

Diez-Fraile, A., Meyer, E., Burvenich, C., 2003a. Sympathoadrenal and immune system activation during the periparturient period and their association with bovine coliform mastitis. A review. Veterinary Quarterly 25, 31-44.

Diez-Fraile, A., Meyer, E., Duchateau, L., Burvenich, C., 2003b. L-selectin and β2-integrin expression on circulating bovine polymorphonuclear leukocytes during endotoxin mastitis. Journal of Dairy Science 86, 2334-2342.

Fernandez-Velasco, M., Prieto, P., Terron, V., Benito, G., Flores, J.M., Delgado, C., Zaragoza, C., Lavin, B., Gomez-Parrizas, M., Lopez-Collazo, E., et al., 2012. NOD1 activation induces cardiac dysfunction and modulates cardiac fibrosis and cardiomyocyte apoptosis. PLoS One 7, e45260.

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. Journal of Immunology 174, 3633-3642.

Fritz, J.H., Girardin, S.E., Fitting, C., Werts, C., Mengin-Lecreulx, D., Caroff, M., Cavaillon, J.M., Philpott, D.J., Adib-Conquy, M., 2005. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. European Journal of Immunology 35, 2459-2470.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J., Sansonetti, P.J., 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide

(MDP) detection. Journal of Biological Chemistry 278, 8869-8872.

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

Huang, Q., Liu, D., Majewski, P., Schulte, L.C., Korn, J.M., Young, R.A., Lander, E.S., Hacohen, N., 2001. The plasticity of dendritic cell responses to pathogens and their components. Science 294, 870-875.

Inohara, N., Ogura, Y., Chen, F.F., Muto, A., Nunez, G., 2001. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. Journal of Biological Chemistry 276, 2551-2554.

Kawai, T., Akira, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. International Immunology 21, 317-337.

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., et al., 2011. Identification of inhibitors of NOD1-induced nuclear factor-kappaB activation. ACS Medicinal Chemistry Letters 2, 780-785.

Kim, Y.G., Park, J.H., Shaw, M.H., Franchi, L., Inohara, N., Nunez, G., 2008. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. Immunity 28, 246-257.

Klesius, P.H., Chambers, W.H., Schultz, R.D., 1984. Effect of bacterial lipopolysaccharide on bovine polymorphonuclear neutrophil migration in vitro. Veterinary Immunology and Immunopathology 7, 239-244.

McDonald, J.U., Cortini, A., Rosas, M., Fossati-Jimack, L., Ling, G.S., Lewis, K.J., Dewitt, S., Liddiard, K., Brown, G.D., Jones, S.A., et al., 2011. In vivo functional analysis and genetic modification of in vitro-derived mouse neutrophils. FASEB Journal 25, 1972-1982.

Mogensen, T.H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical Microbiology Reviews 22, 240-273.

Muhlbauer, M., Cheely, A.W., Yenugu, S., Jobin, C., 2008. Regulation and functional impact of lipopolysaccharide induced Nod2 gene expression in the murine epididymal epithelial cell line PC1. Immunology 124, 256-264.

Nathan, C., 2006. Neutrophils and immunity: Challenges and opportunities. Nature Reviews Immunology 6, 173-182.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C., 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. Journal of Mammary Gland

Biology and Neoplasia 7, 109-121.

Revelo, X.S., Waldron, M.R., 2012. In vitro effects of *Escherichia coli* lipopolysaccharide on the function and gene expression of neutrophils isolated from the blood of dairy cows. Journal of Dairy Science 95, 2422-2441.

Sabroe, I., Prince, L.R., Jones, E.C., Horsburgh, M.J., Foster, S.J., Vogel, S.N., Dower, S.K., Whyte, M.K., 2003. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. Journal of Immunology 170, 5268-5275.

Savill, J., Dransfield, I., Gregory, C., Haslett, C., 2002. A blast from the past: Clearance of apoptotic cells regulates immune responses. Nature Reviews Immunology 2, 965-975.

Shigeoka, A.A., Kambo, A., Mathison, J.C., King, A.J., Hall, W.F., Da, S.C.J., Ulevitch, R.J., McKay, D.B., 2010. Nod1 and nod2 are expressed in human and murine renal tubular epithelial cells and participate in renal ischemia reperfusion injury. Journal of Immunology 184, 2297-2304.

Sohn, E.J., Paape, M.J., Bannerman, D.D., Connor, E.E., Fetterer, R.H., Peters, R.R., 2007a. Shedding of sCD14 by bovine neutrophils following activation with bacterial lipopolysaccharide results in down-regulation of IL-8. Veterinary Research 38, 95-108.

Sohn, E.J., Paape, M.J., Connor, E.E., Bannerman, D.D., Fetterer, R.H., Peters, R.R., 2007b. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-α, IL-1β, IL-12 and IFN-γ. Veterinary Research 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? Veterinary Immunology and Immunopathology 149, 151-156.

Stevens, M.G., Peelman, L.J., De Spiegeleer, B., Pezeshki, A., Van De Walle, G.R., Duchateau, L., Burvenich, C., 2011. Differential gene expression of the toll-like receptor-4 cascade and neutrophil function in early- and mid-lactating dairy cows. Journal of Dairy Science 94, 1277-1288.

Strober, W., Murray, P.J., Kitani, A., Watanabe, T., 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. Nature Reviews Immunology 6, 9-20.

Takahashi, Y., Isuzugawa, K., Murase, Y., Imai, M., Yamamoto, S., Iizuka, M., Akira, S., Bahr, G.M., Momotani, E., Hori, M., et al., 2006. Up-regulation of NOD1 and NOD2 through TLR4 and TNF-α in LPS-treated murine macrophages. Journal of Veterinary Medical Science 68, 471-478.

Tamassia, N., Calzetti, F., Ear, T., Cloutier, A., Gasperini, S., Bazzoni, F., McDonald, P.P., Cassatella, M.A., 2007. Molecular mechanisms underlying the synergistic induction of

CXCL10 by LPS and IFN-γ in human neutrophils. European Journal of Immunology 37, 2627-2634.

Tan, X., Li, W.W., Guo, J., Zhou, J.Y., 2012. Down-regulation of NOD1 in neutrophils of periparturient dairy cows. Veterinary Immunology and Immunopathology 150, 133-139.

Tecchio, C., Micheletti, A., Cassatella, M.A., 2014. Neutrophil-derived cytokines: Facts beyond expression. Frontiers in Immunology 5, 508.

Tharwat, M., 2011. Accelerated neutrophil apoptosis in cows affected with acute mastitis. Journal of Agricultural and Veterinary Sciences 4, 125-134.

Tirsoaga, A., Novikov, A., Adib-Conquy, M., Werts, C., Fitting, C., Cavaillon, J.M., Caroff, M., 2007. Simple method for repurification of endotoxins for biological use. Applied and Environmental Microbiology, 73, 1803-1808.

Tourneur, E., Ben, M.S., Chassin, C., Bens, M., Goujon, J.M., Charles, N., Pellefigues, C., Aloulou, M., Hertig, A., Monteiro, R.C. et al., 2013. Cyclosporine A impairs nucleotide binding oligomerization domain (Nod1)-mediated innate antibacterial renal defenses in mice and human transplant recipients. PLoS Pathogens 9, e1003152.

van Heel, D.A., Ghosh, S., Butler, M., Hunt, K., Foxwell, B.M., Mengin-Lecreulx, D., Playford, R.J., 2005. Synergistic enhancement of Toll-like receptor responses by NOD1 activation. European Journal of Immunology 35, 2471-2476.

Vangroenweghe, F., Lamote, I., Burvenich, C., 2005. Physiology of the periparturient period and its relation to severity of clinical mastitis. Domestic Animal Endocrinology 29, 283-293.

Worku, M., Morris, A., 2009. Binding of different forms of lipopolysaccharide and gene expression in bovine blood neutrophils. Journal of Dairy Science 92, 3185-3193.

Xing, L., Remick, D.G., 2003. Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils. Shock 20, 10-16.

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. Journal of Leukocyte Biology 75, 358-372.

Zheng, W., Zheng, X., Liu, S., Ouyang, H., Levitt, R.C., Candiotti, K.A., Hao, S., 2012. TNFα and IL-1β are mediated by both TLR4 and Nod1 pathways in the cultured HAPI cells stimulated by LPS. Biochemical and Biophysical Research Communications 420, 762-767.

Zoldan, K., Moellmer, T., Schneider, J., Fueldner, C., Knauer, J., Lehmann, J., 2014. Increase of CD25 expression on bovine neutrophils correlates with disease severity in post-partum and early lactating dairy cows. Developmental and Comparative Immunology 47, 254-263.

555 **Figure legends**

556

557 Fig. 1. Phosphorylation of inhibitor of NF-κBα (IκBα). (A) Isolated neutrophils were incubated with
558 or without ML130 (30 μmol/L) for 2 h, followed by exposure to crude lipopolysaccharide (cLPS)
559 (100 ng/mL) or NOD1 agonist iE-DAP (10 μg/mL) for a further 4 h. Protein lysates were made
560 from the cells and then probed by Western blot analysis using anti-phospho (p)-IκBα. (B)
561 Densitometry was performed on pIκBα/β-actin Western blots from 3-4 replicates using Quantity
562 One software. Data (mean ± standard deviation, SD) are presented as fold change relative to the
563 basal level (control cells). Data are from individual heifers (n = 3). ***P <0.001 vs. basal ; $^{###}P$
564 <0.001 between groups.

565

566 Fig. 2. Effect of ML130 on crude lipopolysaccharide (cLPS)-induced NOD1 expression. (A)
567 Western blot analysis showing the protein levels of NOD1in different treatments. (B) Protein was
568 quantified using the densitometry function of Quantity One software, normalised to β-actin within
569 the same sample and expressed as fold change relative to the basal level (control cells). All Western
570 blots were generated in three replicates. Data (mean ± standard deviation, SD) are from individual
571 heifers (n = 3). *P <0.05 vs. basal; $^{#}P$ <0.05 vs. ML130.

572

573 Fig. 3. Effect of inhibition of NOD1-mediated NF-κB activation on the expression of (A)
574 pro-inflammatory cytokines, (B) chemokines and (C) adhesion molecules in crude
575 lipopolysaccharide (cLPS)-challenged neutrophils. RNA was quantified using SYBR Green-based
576 quantitative PCR (qPCR) and data were analysed using the Pfaffl method. Results are expressed as
577 fold change relative to the basal level (control cells). Data are mean ± standard deviation (SD, n = 6)

578 from one experiment representative of three.

579

580 Fig. 4. Crude lipopolysaccharide (cLPS)-induced neutrophil migration involves NOD1-dependent
581 NF-κB activation. Cell migration was measured using the Transwell system. The data presented are
582 mean ± standard deviation (SD) of six heifers. * P <0.05 and ***P <0.001 vs. basal; $^{\#\#}P$ <0.01 vs.
583 cLPS.

584

585 Fig. 5. Impairment of intracellular NOD1/NF-κB pathway promotes cell death in crude
586 lipopolysaccharide (cLPS)-challenged neutrophils. Apoptosis was analysed using fluorescein
587 isothiocyanate (FITC)-labelled annexin-V in combination with propidium iodide (PI) staining. (A)
588 Representative fluorescein activated cell sorting (FACS) plot of apoptotic cells (lower right). (B)
589 Bar chart corresponds to the percentage of FITC-annexin-V-labelled cells (mean ± standard
590 deviation, SD, of six heifers). **P <0.01 vs. cLPS.

591

592 Fig. 6. Phagocytosis and oxidative burst activity of neutrophils in whole blood. (A) Representative
593 fluorescent activated cell sorting (FACS) plot showing flow cytometric detection of phagocytosis by
594 neutrophils treated with cLPS (grey fill), ML130 + cLPS (black line) or left untreated (grey line).
595 (B) Flow cytometry histogram showing rhodamine 123 fluorescence corresponding to oxidative
596 burst activity. Neutrophil phagocytosis and oxidative burst are indicated by phagocytic index (C)
597 and oxidative burst index (D). Data are given as mean ± standard deviation (SD) of five heifers.
598 ***P <0.001 vs. basal (control cells); $^{\#}P$ <0.05 and $^{\#\#}P$ <0.01 vs. cLPS.

599

Figure 1

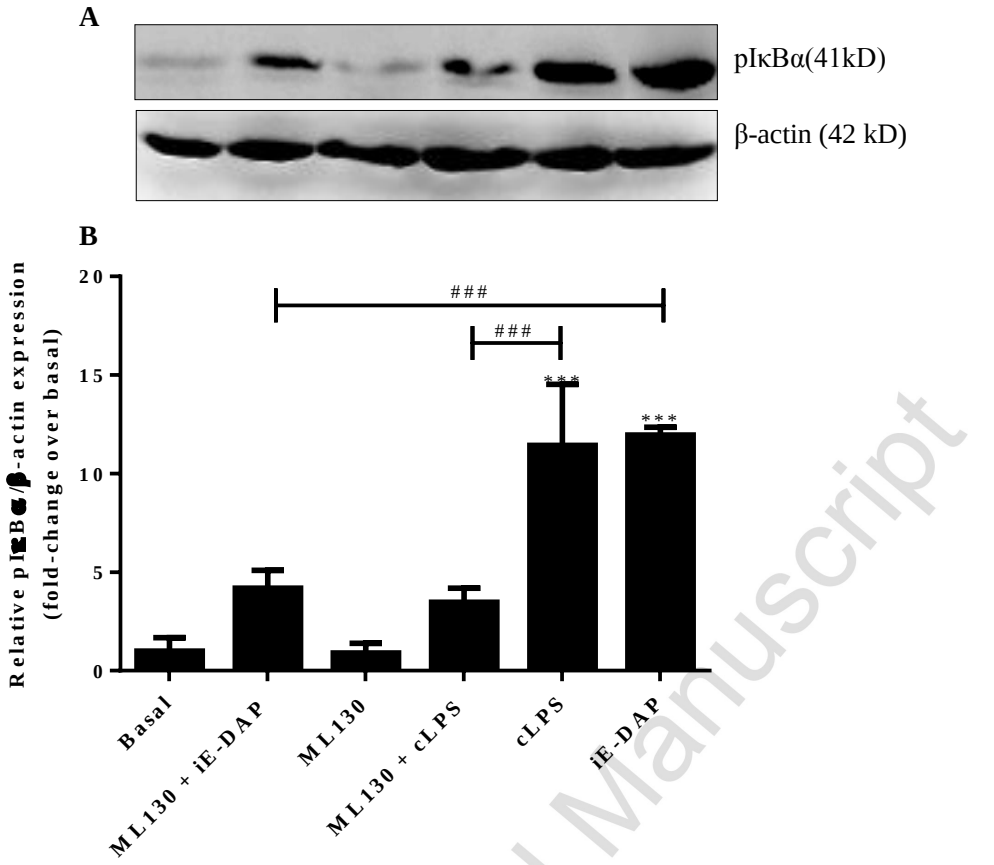

Figure 2

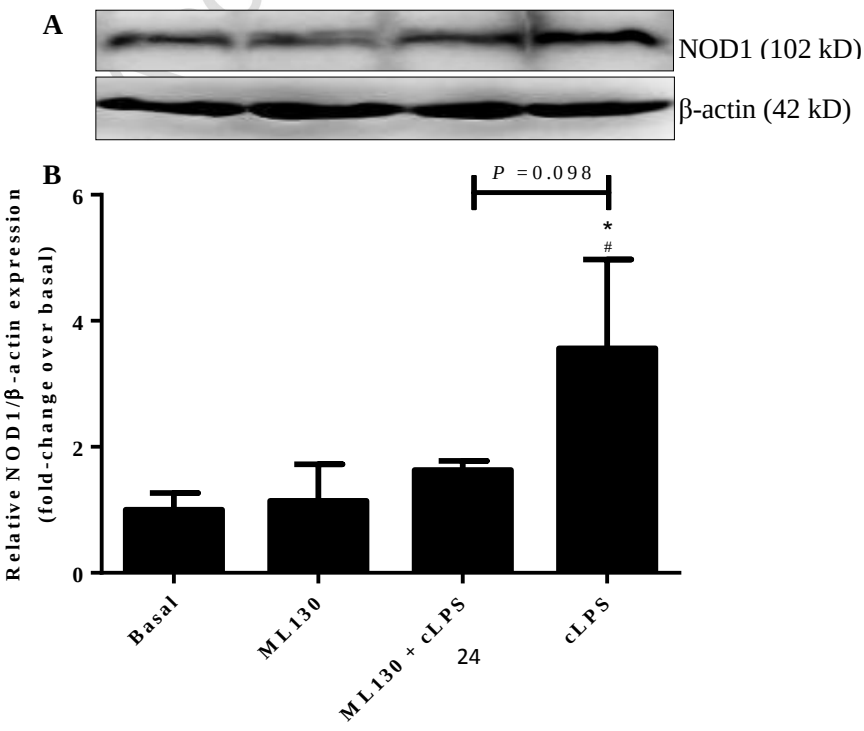

Figure 3

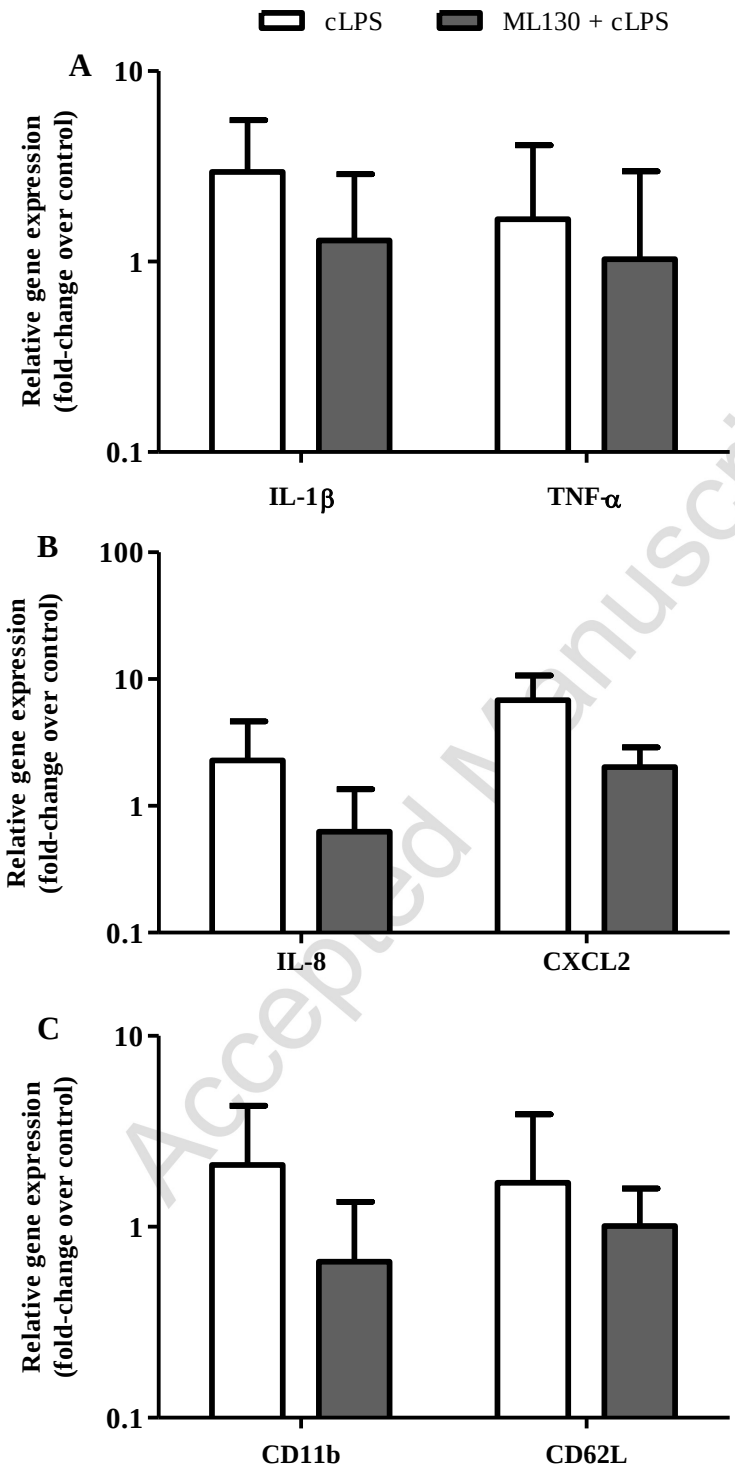

Figure 4

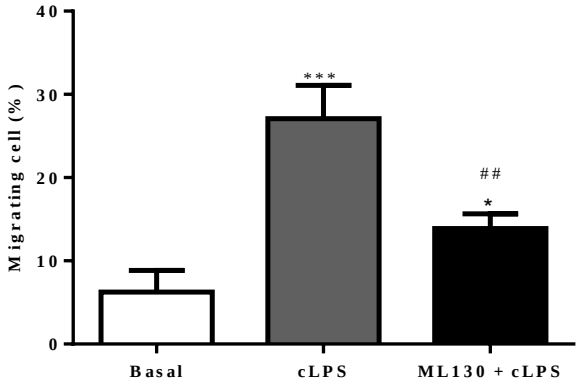

Figure 5

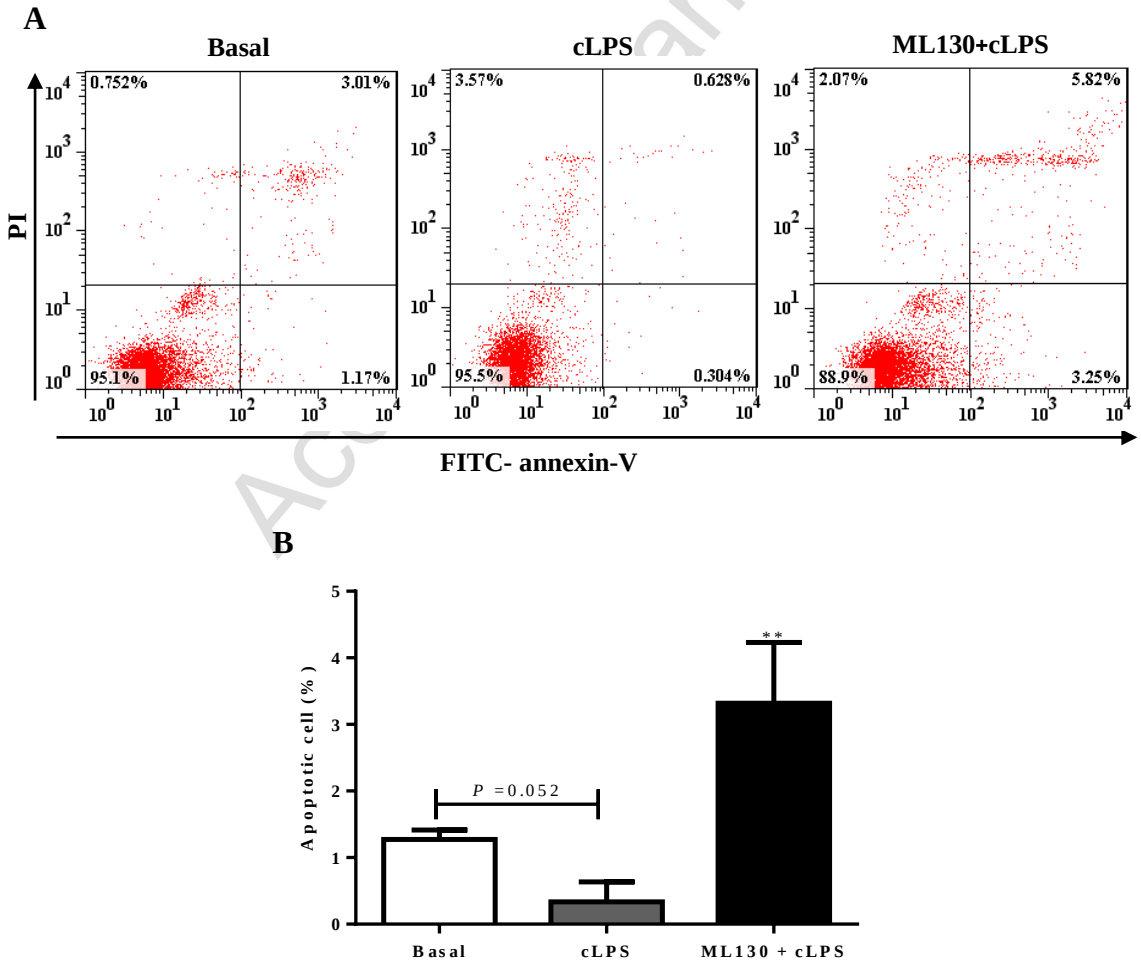

Figure 6