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Effect of the synthetic Toll-like receptor ligands LPS, Pam_3CSK_4, HKLM and FSL-1 in the function of bovine polymorphonuclear neutrophils

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Highlights

- TLR ligands induces morphology changes and the phagocytosis activity in bovine PMN
- Bovine PMN express TLR2 and TLR4 at the membrane surface
- Pam_3CSK_4 induces calcium influx, ROS production and MMP-9 secretion
- The ROS production induced by Pam_3CSK_4 was blocked by the SOCE inhibitor 2-APB
- Incubation with TLR ligands does not affect the apoptosis of bovine PMN

Abstract

Toll-like receptors (TLR) are a family of pattern recognition receptors that sense microbial associated molecular patterns (MAMP) such as microbial membrane components and nucleic acids of bacterial origin. Polymorphonuclear neutrophils (PMN) are the first cell of the innate immune system to arrive at the site of infection or injury and elicit oxidative and non-oxidative microbicidal mechanisms. Observations in human and mouse suggest that TLR ligands can induce direct responses in PMN. So far, there is no information of the effect of synthetic TLR ligands on the response of bovine PMN. The objective of this study was to evaluate the functional response of bovine PMN incubated with four synthetic TLR ligands: ultrapure LPS (TLR4), Pam_3CSK_4 (TLR2/1), HKLM (TLR2) and FSL-1 (TLR2/6). The results show that all the ligands increment cells size as identified by changes in the FSC-SSC as part of the flow cytometric analysis. Interestingly, only Pam_3CSK_4 consistently induced a calcium influx, increased ROS production and secretion of gelatinase granules, whereas no response was seen using other ligands. Furthermore, exposure of bovine PMN to ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1 for 24 hours did

40 not impact on apoptosis of these cells. Our data provide evidence for a selective response of
41 bovine PMNs to TLR ligands.

42

43 Keywords: PMN, ROS, Toll-like receptors, Bovine, Innate immunity.

44 Abbreviations: SOCE: Store Operated Calcium Entry. AUC: Area under the curve. RFU:
45 Relative fluorescence units. RLU: Relative luminescence units.

46

1. Introduction

Polymorphonuclear neutrophils (PMN) are the first line of cellular defense against bacterial and fungal agents (Yu and Czuprynski, 1996), and rapidly arrive at the site of injury or infection, recognizing and attempting to resolve the infection through various antimicrobial mechanisms (Segal, 2005). These include phagocytosis, reactive oxygen species (ROS) production, secretion of granules that contains several antimicrobial proteins (Borregaard and Cowland, 1997; Paape et al., 2003), the ability to cast neutrophil extracellular traps (NET) (Behrendt et al., 2010; Brinkmann et al., 2004) and chemokine/cytokine production that induces the arrival of leukocytes to the site of infection (Hammond et al., 1995). The complex process of mounting these steps of the inflammatory response must be tightly regulated in order to avoid subsequent damages to host cells by overshooting responses (Nathan, 2006). In this context, the detection and sensing of the molecules produced in the first steps of infection or injury (i.e. chemoattractants) and microbial associated molecular patterns (MAMPs) are relevant.

Toll like receptors (TLR) are a family of pattern recognition receptors (PRR) which sensing different MAMPs that includes lipoproteins, lipopolysaccharide, flagellin and nucleic acids from bacterial origin. In addition, these receptors bind endogenous ligands such as heat shock proteins (HSP) and structural molecules such as fibrinogen, heparan sulfate and soluble hyaluronan. These molecules are constituents of the extracellular matrix and are termed as danger-associated molecular pattern (DAMP), as their recognition by TLRs is associated with inflammatory response during tissue damage and tissue repair (Kawai and Akira, 2010). The intracellular signaling pathways activated after TLR-ligand binding have been classified in the MyD88-dependent pathway and the MyD88-independent or TRIF-

dependent pathway. The MyD88 dependent pathway involves the recruitment of IL-1 receptor-associated kinases: IRAK4, IRAK1, IRAK2, TRAF-6 and the activation of the MAPK pathway. This cascade of events leads to the translocation of the nuclear transcription factor NF-κB and the increased expression of proinflammatory genes as COX-2, CXCL-8 and IL-6. This pathway is essential for all TLRs with the exception of TLR3. The TRIF-dependent pathway also activates NF-κB and, in addition, the interferon regulatory transcription factor 3 (IRF-3) leading to an increased transcription of type I interferons (Akira, 2011; Kawai and Akira, 2010).

To date, 10 TLRs has been identified in bovines (McGuire et al., 2006) and the presence of these receptors in cells of the innate immune system permits an initial response that is amplified by the adaptive immune system. However, less is known about the direct activation of TLRs in cells of the innate immune system. In bovines, the TLRs has been associated with the recognition of *Mycobacterium (M.) tuberculosis* and *M. bovis* by macrophages (Werling et al., 2006), infectious agents involved in bovine respiratory disease (Hodgson et al., 2005) and *E. coli* mediated mastitis (De Schepper et al., 2008). PMN have been described to express numerous PRRs (Brown, 2006; Chavakis et al., 2003), and specifically in bovines the detection of mRNA for TLR1, TLR2, TLR4, TLR6, TLR7 and TLR10, but not TLR3, TLR5, TLR8, TLR9 has been reported previously (Conejeros et al., 2011). Exposure of human neutrophils to TLR agonist triggered or primed cytokine release, superoxide generation, and L-selectin shedding, while inhibiting chemotaxis to CXCL8 and increasing phagocytosis of opsonized latex beads. Some of these effects where amplified when PMN where pre-incubated with GM-CSF (Hayashi et al., 2003). In addition, direct activation of TLR2 or TLR4 with synthetic TLR agonist induces

changes on the expression of adhesion molecules, CXCL8 production, ROS generation and random migration (Aomatsu et al., 2008; Sabroe et al., 2003). In contrast, there is no information regarding the effect of synthetic TLR ligands in the direct activation of bovine PMN. However, bovine PMN have been show to respond with ROS production to the dectin-1/TLR 2 ligand zymosan (Conejeros et al., 2011), suggesting that the incubation of bovine PMN with TLR ligands can induce the activation of these cells. Intracellular calcium concentration governs several functional responses in bovine PMN as ROS production, MMP-9 secretion, CD11b and CD63 expression, chemotaxis and F-actin polymerization (Conejeros et al., 2012). One of the mechanisms that explain the rise of intracellular calcium concentration is the store operated calcium entry (SOCE). This retrograde process involves the opening of membrane calcium channels in response to the recognition of inositol 1,4,5-triphosphate (IP_3) by the corresponding receptors (IP_3R) in the endoplasmic reticulum. The rise in cytoplasmic IP_3 is produced by PI3K in response to the bind of the ligand with the corresponding G-coupled receptor located at the plasmatic membrane. These ligands include different inflammatory mediators as platelet activating factor (PAF), LTB4, C5a and CXCL8. The increasing evidence regarding the modulation of oxidative and non-oxidative responses in bovine PMN supports the therapeutic potential of this pathway in inflammatory diseases with neutrophil infiltration in cattle (Burgos et al., 2011).

Given the lack of knowledge regarding the activation of bovine PMN by TLR ligands, we intended to study functional responses associated with neutrophil activation in the presence of synthetic ligands for TLR 2/1: Pam_3CSK_4, TLR 4: ultrapure LPS, TLR2: HKLM and TLR2/6: FSL-1. The evaluated parameters in bovine PMN exposed to these ligands were

size and shape changes, phagocytosis, intracellular calcium concentration, ROS production, gelatinase granules secretion, CD11b and L-selectin and apoptosis. In addition and due to the evidence that supports the role of the rise in intracellular calcium concentration in the first steps of activation of bovine PMN, the PMN were exposed to Pam_3CSK_4 in the presence of the SOCE inhibitors 2-APB 50 µM and MRS1845 15 µM.

2. Material and Methods

2.1. Animals and samples

Blood samples were obtained by jugular venipuncture of healthy adult Holstein Friesian heifers from one of the herds of the University Austral of Chile, Valdivia, Chile. The cattle were maintained in the ruminant section of the Veterinary Hospital on a grass diet *ad libitum* with grain supplementation. The blood was collected in ACD tubes (Becton Dickinson, NJ, USA) and maintained at room temperature (RT) for less than 20 min prior to the beginning of the assays. All experiments were conducted in accordance with institutional review board-approved protocols.

2.2. Synthetic TLR ligands and SOCE inhibitors

Pam_3CSK_4 and ultrapure LPS from *E. coli* serotype EH100 Ra were obtained from Enzo Lifesciences (NY, USA), diluted in nanopure water to stock concentration of 1 mg/ml and stored at -20 °C. HKLM was obtained from KPL (MA, USA) at stock concentration of $5x10^{10}$ cells/ml and stored in aliquots at -20 °C until use. FSL-1 was acquired from EMC microcollections (Tübingen, Germany), suspended in nanopure water to a stock concentration of 1mg/ml and stored at -20 °C until further use. 2-APB was acquired from Sigma Aldrich (St. Louis, MO, USA), suspended in DMSO 100% at stock concentration of

100 mM and stored at -80 °C. MRS1845 was obtained from Tocris Bioscience (Bristol, UK), suspended at stock concentration of 10 mM in DMSO 100% and stored at -20 °C.

2.3. Isolation of bovine PMN

Blood in ACD tubes (Becton Dickinson, NJ, USA) was used for the isolation of bovine PMN according to the method described by Roth and Kaeberle (Roth and Kaeberle, 1981) with minor modifications. After collection, the blood was gently rocked for 5 min and then centrifuged at 1000*g* for 20 min at 20 °C. The plasma and the buffy coat were carefully aspirated, and the remaining red blood cells (RBC) and PMN pellet in the bottom of the tube was suspended in Hank's balanced salt solution (HBSS). The mixture was transferred to a new sterile 15 ml polypropylene tube (BD, NJ, USA) and centrifuged again at 1000*g* for 20 min at 20 °C. The supernatant was aspirated, discarded and the RBC were removed by a flash hypotonic lysis with 1 volume of cold phosphate buffered water solution containing 5.5mM NaH_2PO_4 , 8.4 mM HK_2PO_4 at pH 7.2. After 1 min of RBC lysis a 2 volumes of hypertonic phosphate buffer containing 5.5 mM NaH_2PO4, 8.4 mM HK_2PO_4, 0.46 M NaCl and pH 7.2 was added to recover the isotonicity and the tubes were centrifuged at 600*g* for 10min at 20 °C. This RBC lysis step was repeated once and after the cells were washed three times with HBSS with centrifugation steps of 500*g* for 10 min at 20 °C. The purity of the cell preparation was assessed by flow cytometry and a purity ≥ 90% was a condition for continuing with the assays. The cell preparation was maintained on ice until the experiments were performed.

2.4. Size and morphology changes of bovine PMN

Isolated PMN were suspended in HBSS containing 0.9 mM $CaCl_2$ at a final concentration of $1x10^7$ cells/ml. For the assays, 2.5×10^4 PMN were mixed with 1 µg/ml of ultrapure LPS, 10 µg/ml of Pam_3CSK_4 , 108 cells/ml of HKLM, or 1µg/ml of FSL-1 in cytometer tubes (BD Biosciences, San Diego, CA), respectively. The size changes in the forward light scatter (FSC) axis of the PMN gate were registered after 10 sec of incubation by flow cytometry recording 10.000 events. The results are presented as side light scatter (SSC) versus FSC. The data was analyzed to obtain histograms of FSC changes using the Flow Jo 7.2.1 software (www.flowjo.com Tree Star, Inc., USA)

2.5. Phagocytosis assay

Whole blood was incubated with the TLR ligands for 15 min and the percentage of positive PMN to phagocytosis was determined using the pHrodo phagocytosis kit for flow cytometry containing bioparticles derived from *S. aureus* (Life Technologies, CA, USA) following the manufacturer's instructions. 10.000 events were recorded in a FACS Canto II flow cytometer and analyzed with the FACSDIVA 6.1 software (BD Biosciences, CA, USA)

2.6. Intracellular calcium measurement

PMN were suspended in HBSS at a concentration of 1×10^7 cells/ml and incubated with 1µM FLUO-4/AM (Molecular Probes, Oregon, USA) for 30 min at 37°C. The cells were washed two times in HBSS, and suspended at 1×10^7 cells/ml in HBSS containing 0.9 mM $CaCl_2$. For the assays, $1x10^6$ cells per well were used and stimulated with increasing concentrations of ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1. For the inhibition assays with 2-APB 50 µM and MRS1845 15 µM the cells were incubated for 15 min before the

addition of the Pam_3CSK_4 10 µg/ml. The Ca^{2+} influx was measured at 37 °C in a Varioskan microplate reader (Thermo, USA) at 488 nm and 525 nm, excitation and emission wavelengths respectively. The area under the curve (AUC) after 400 sec of stimulation for each treatment was determined and plotted as concentration in logarithmic scale x AUC. Each point represents the mean ± SEM.

2.7. ROS production measurement

Before the treatments, a total of 1 x 10^6 PMN per well were incubated with luminol 80µM for 5 min at 37°C. Basal production of ROS was registered and increased concentrations of ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1 were added to the corresponding wells. The light emission produced by the reaction of luminol and H_2O_2 was measured over the time in a Varioskan microplate reader (Thermo, USA). For the inhibition assays the cells were incubated for 15 min before the addition of luminol with 2-APB 50µM and MRS1845 15µM. The AUC of the chemoluminiscence curves registered was calculated and represented as concentration in logarithmic scale x concentration. Each point represents the mean ± SEM.

2.8. Determination of MMP-9 activity

A total of 1 x 10^6 PMN in 500 µl HBSS containing 0.9 mM $CaCl_2$ were incubated with increasing concentrations of ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1 for 120 min at 37°C. For the inhibition assays the cells were incubated for 15 min before the addition of the Pam_3CSK_4 10 µg/ml with 2-APB 50µM and MRS184515 µM. After incubation, the cells were centrifuged at 600*g* for 6 min at 20 ºC. A total of 300 µl of the supernatant was recovered and used for gelatinase activity analysis. Substrate gel electrophoresis was performed using the method described by Li (Li et al., 1999), with minor modifications.

Briefly, 10 µl of supernatant was loaded on 10% polyacrylamide gels (0.75 mm thick) containing 0.28% gelatin. The gels were run at 200 V for 1 h in a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad Laboratories, Richmond, CA) and then soaked twice in 2.5% Triton X-100 in distilled water on a shaker at RT for 30 min. Then, the gels were soaked in reaction buffer consisting of 100 mM Tris (pH 7.5) and 10 mM $CaCl_2$ at 37 ºC overnight. The gels were stained in 0.5% Coomassie Brilliant Blue R-250 (Winkler, Santiago, Chile) in acetic acid:methanol:water (1:3:6). Evidence of enzymatic activity was determined by non-staining areas in which the gelatin was degraded. The calculation of the apparent molecular masses of the gelatinolytic bands was made using a reference to a standard pre-stained molecular mass marker (Fermentas International Inc., Canada). To measure the activity, the gels were digitalized, and the intensity of the bands was determined using ImageJ 1.46r software. The results are presented in bar graphs as normalized densitometry units x concentration.

2.9. Flow cytometric analysis of CD11b and L-selectin

200 µl of whole blood were incubated with of ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1 for 30 min at 37 °C in 12x75 mm polypropylene round bottom tubes (BD, NJ, USA). Then, 1 ml of lysing buffer (BD Pharm Lyse, CA, USA) was added with each tube, with gently vortex just after the addition of the buffer. The mixture was incubated at RT, protected from light, for 15 min. After, a centrifugation step of 200*g* for 5 min at RT was performed and the supernatant was discarded carefully. Then, 2 ml of stain buffer (BD Pharmingen, CA, USA) was added to wash the cells and the solution was centrifuged at 200*g* for 5 min at RT. This step was repeated once and the cells were suspended in 100µl of stain buffer for immunofluorescent staining. For CD11b 5 µl (0.2 mg/ml) of human CD11b antibody

coupled with allophycocyanin (APC; clone M1/70 from BD Pharmingen, CA, USA) was added. For the L-selectin immunostaining 10µl of the L-selectin antibody coupled with phycoerytrin was added (PE; clone DREG-56 from BD Pharmingen, CA, USA). The tubes were incubated for 20 min at RT in darkness and washed two times with 1 ml of stain buffer with centrifugation steps of 200*g* for 5 min at RT. Finally, the cells were suspended in 300µl of stain buffer and analyzed by flow cytometry (FACSCanto II, BD, CA, USA). 10,000 events were registered in the neutrophil gate population. These antibodies show cross-reactivity with bovine PMN and have been described previously for us and other authors (Conejeros et al., 2012; Swain et al., 1998).

To confirm the observed results another antibody, specific for bovine CD11b was used (monoclonal antibody center, WSU, Washington, USA) in isolated PMN. $0.5x10^6$ PMN were incubated with the TLR ligands for 30 min and after two washing steps with stain buffer as described above were incubated with 15 µg/ml an antibody that is specific for bovine CD11b (monoclonal antibody center, WSU, Washington, USA) protected from light for 20 min on ice. After a two washing steps, 1/100 dilution of secondary anti-mouse IgG antibody coupled to Alexa 488 (Life technologies, CA, USA) was added and the solution was incubated for 20 min in the dark. After incubation, the cells were centrifuged at 200*g* for 5 min and washed two times with stain buffer. Finally, the cells were gently suspended in 300 µl for flow cytometry analysis. Data analyses were performed using FACSDIVA (BD Biosciences, CA, USA) and FlowJo 7.1 (www.flowjo.com Tree Star, Inc., USA) software.

2.10. Flow cytometric analysis of CD11b, TLR2 and TLR4

Bovine PMN from 3 animals were examined for co-expression of CD11b with either TLR2 or TLR4. 0.5 x 10^6 of isolated bovine PMN per antibody combination were placed in a round bottomed 96-well plate and centrifuged at 200*g* for 2 min before suspending cells in 40 µl of stain buffer. 5 µl of each antibody (or stain buffer) was added corresponding antibody combinations as follows: (1) no antibody control, (2) isotype controls (IgG2b coupled with Fluorescein isothiocyanate (FITC) (AbD Serotec, Oxford, UK) and IgG2a coupled with Alexa 647 (Life Technologies, Paisley, UK), for TLR4 and huCAL Fab-dHLX-MH (AbD Serotec, Oxford, UK) for TLR2, (3) mouse anti-bovine CD11b IgG2b coupled to FITC and human anti-bovine TLR2 huCAL Fab bivalent coupled to Alexa 647 (both AbD Serotec, Oxford, UK) (Kwong et al., 2011) and (4) mouse anti-human CD11b IgG1 coupled to FITC (AbD Serotec, Oxford, UK) and mouse anti-human TLR4 IgG2a coupled to Alex 647 (Novus Biologicals, Cambridge, UK). Incubation of antibody and cells was allowed to proceed at 4 °C for 30 min in the dark before washing cells with addition of 150 µl of stain buffer and centrifugation at 200*g* for 2 min. Washing was repeated twice more and cells suspended in 400 µl for analysis using a FACS Calibur (BD Biosciences, Oxford UK) running Cell Quest Pro acquiring 10,000 events. Data analyses using were performed using Flow Jo V10 (www.flowjo.com Tree Star, Inc., USA). Further confirmatory experiments were performed using HEK293T cells alone or expressing either bovine TLR2 or bovine TLR4 in combination with bovine MD2. Briefly as above, 0.5 x 10^6 cells were placed a round bottomed 96-well plate and centrifuged at 200g for 2 min before suspending cells in 45 µl of stain buffer. 5 µl of each antibody was added for 30 min at 4°C in the dark; isotype control IgG2a coupled with Alexa 647 (Life Technologies, Paisley, UK) for TLR4 and huCAL Fab-dHLX-MH (Abd Serotec, Oxford, UK) for TLR2,

human anti-bovine TLR2 huCal Fab bivalent coupled to Alexa 647 (AbD Serotec, Oxford, UK) or mouse anti-human TLR4 IgG2a coupled to Alex 647 (Novus Biologicals, Cambridge, UK). Cells were washed and analysis performed as above. Staining was observed for HEK293T cells expressing bovine TLR2 or bovine TLR4/MD2 only, validating further the cross-reactivity of these antibodies.

2.11. Assessment of live, apoptotic and dead PMN

0.5×10^6 isolated PMN dispensed in a 96-well microplate were incubated for 24 hours at 37°C and 5% CO_2 in 200 µl of RPMI-medium with increasing concentrations of ultrapure LPS, Pam_3CSK_4, HKLM or FSL-1. After the incubation, the medium was gently removed and the samples were processed with the annexin V-FITC and PI detection kit I for apoptosis following the manufacturer's protocol (BD Pharmingen, CA, USA).

2.12. Statistical analysis

The results are illustrated as mean ± SEM for at least three independent experiments. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test were performed using GraphPad Prism v5.3 software (GraphPad Software Inc., CA, USA) with a significance level of 5%. For the determination of statistical significant differences in the percentages of phagocytosis-positive PMN a one-tailed Student *t* test was used comparing the control vs ligand conditions.

3. Results

3.1. Effect of synthetic TLR ligands on cell size and morphology of bovine PMN

All TLR ligands used in this study induced a rapid change in cell size and morphology of bovine PMN. As early as 10 sec after the onset of stimulation, a shift along the FSC axis, indicating a shift in cell size, could be observed in the histograms analysis of the gated PMN population when the cells were exposed to ultrapure LPS, Pam_3CSK_4, HKLM and FSL-1 in the specified concentration (Fig 1).

3.2. Exposure of bovine PMN to TLR ligands induces the phagocytosis of *S. aureus* bioparticles

Percentage of phagocytosis-positive PMN was determined using a phagocytosis kit for flow cytometry containing bioparticles derived from *S. aureus* conjugated with the pHrodo dye. The incubation of whole blood with the TLR ligands results in an increase of the PMN positive for phagocytosis compared with the control condition, except for the ultrapure LPS treatment which did not reach significance (Table 1)

3.3. Exposure of bovine PMN to TLR ligands does not affect CD11b expression or L-selectin (CD62L) shedding

Activation of the TLR2 pathway by Pam_3CSK_4 treatment has recently been shown to induce CD11b/CD18 surface expression and ROS production by human neutrophils (Lee et al., 2012). As TLR stimulation in the present experiment impacted in the morphology of bovine PMN after a very short time of exposure, we next wanted to assess whether CD11b expression and L-selectin shedding were also affected. Addition of all TLR ligands to whole blood preparations failed to result in any differences of CD11b or L-selectin expression. In order to investigate if this observation effect was dependent on the antibody, other blood cells or blood components the measure of CD11b was repeated adding the

synthetic TLR ligands to isolated PMN and using another anti-bovine CD11b antibody. However, even using isolated PMN expression levels of CD11b remained unaltered (data not shown).

3.4. TLR2 and TLR4 expression by bovine PMN

Given changes in PMN morphology upon stimulation with synthetic TLR ligands and lack of CD11b- or CD62L-shedding we examined the expression of TLR2 and TLR4 cell surface protein levels. While various TLR mRNA has been previously reported for bovine PMN (Conejeros et al., 2011), little has been described for cell surface expression largely based upon availability of cross-reactive antibodies. Here we show by flow cytometry that $CD11b^+$ bovine PMN express both TLR2 and TLR4 compared to isotype controls (Fig 2A). Antibody cross-reactivity was further assessed using HEK293T cells expressing either bovine TLR2 or bovine TLR4/MD2. HEK293T cells expressing bovine TLR2 or bovineTLR4/MD2 but not HEK293T cells alone were found to be positive for TLR2 or TLR4 respectively (Fig 2B).

3.5. Stimulation of PMN with a synthetic TLR2 ligand results in increased intracellular calcium concentration and ROS production

We were recently able to show that zymosan, a dectin-1/TLR2 ligand, induced ROS, but not RNS production in a CD11b-, but not dectin-1-dependent manner (Conejeros et al., 2011). As the initial observations supported that TLR ligands induces the rapid activation of isolated bovine PMN, we investigated next whether TLR-ligand interaction increased intracellular calcium concentration in bovine PMN. Indeed, cells treated with Pam_3CSK_4 at 10µg/ml, but not in lower concentrations showed an increase in intracellular calcium

concentration ($p < 0.05$). No changes in intracellular calcium concentration were observed when cells were exposed to different concentrations of ultrapure LPS, HKLM and FSL-1 (Fig 3A). As intracellular calcium concentrations may also impact on ROS production, we assessed next whether exposure of bovine PMN to TLR ligands can trigger ROS production. Similar as before, ROS production was increased significantly when cells were exposed to Pam_3CSK_4 at a concentration of 10µg/ml, but not at lower concentrations. Surprisingly, FSL-1 1µg/ml also induced significantly ($p < 0.05$) ROS production, however at a later time-point compared to Pam_3CSK_4. No effect was observed when cells were exposed to increasing concentrations of ultrapure LPS or HKLM (Fig 3B). Representative registries of the results for calcium influx and ROS production are shown in the appendix (Fig A.2)

3.6. Pam_3CSK_4-induced MMP9 secretion partially depends on ROS production

As ROS has been shown to up-regulate MMP-9 (gelatinase) expression via a MAPK-AP-1 dependent signaling pathway in human PMN (Ehrenfeld et al., 2009), we determined next whether MMP-9 activity was increased in supernatants of treated cells in order to determine the effect of the synthetic TLR ligands on the secretion of gelatinase granules. High concentration of Pam_3CSK_4, but none of the other TLR ligands tested induced a significant increase in the secretion of MMP-9 ($p < 0.05$) as observed in the zymography assay after 120 min of exposure (Fig 4).

To assess whether the effect of Pam_3CSK_4 on calcium influx, ROS production and MMP-9 secretion was a direct effect, two SOCE inhibitors: 2-APB and MRS1845 were used to further investigate the observation. Indeed, pre-incubation of cells with both inhibitors prior

358 to Pam_3CSK_4 exposure partially inhibited the calcium influx ($p < 0.05$; Fig 5A). Further,
359 the effect of SOCE inhibitors on ROS production induced by Pam_3CSK_4 was tested. 2-APB
360 almost completely inhibited ROS production induced by Pam_3CSK_4 ($p < 0.05$), but not
361 MRS1845 (Fig 5B). Astonishingly, MMP-9 secretion from bovine PMN induced by
362 Pam_3CSK_4 was unaffected by the treatment with either 2-APB or MRS1845 (Fig 5C).
363 Representative diagrams of the obtained registries for calcium influx and ROS production
364 from which the area under the curve (AUC) was calculated are shown in (Fig A.3)

365 **3.7. Stimulation with TLR ligands does not induce apoptosis in bovine PMN**

366 Exposure to bacteria and subsequent phagocytosis has been shown to subsequently induce
367 apoptosis in human PMN (for review see (McCracken and Allen, 2014)). As exposure to
368 some TLR ligands induces ROS production in bovine PMN, which in turn can lead to the
369 induction of cell death (for review see (Geering and Simon, 2011)), we assessed whether
370 stimulation by defined TLR ligands would result in neutrophil apoptosis. PMN were
371 incubated with increasing concentrations of ultrapure LPS, Pam_3CSK_4, HKLM and FSL-1,
372 and analyzed after 24 h by flow cytometry using an AnnexinV/Propidium Iodide kit (BD
373 Biosciences). In these experimental conditions, none of the TLR ligands had an impact on
374 the percentage of live, apoptotic or dead cells ($p > 0.05$) (Fig 6).

375 **4. Discussion**

376 In the present study, we confirmed the expression of TLR2 and TLR4 by flow cytometry on
377 bovine PMN, and assessed the effect of synthetic TLR ligands, namely ultrapure LPS
378 (TLR4), Pam_3CSK_4 (TLR2/1), HKLM (TLR2) and FSL-1 (TLR2/6) on the functions of
379 bovine PMN. Previous reports in human support the hypothesis that TLR ligands can

induce direct activation of PMN, resulting in increased bacterial killing and an inflammatory response (Aomatsu et al., 2008; Hayashi et al., 2003; Sabroe et al., 2003). In addition, it is known that PMN from different species have important differences in terms of specificity and magnitude of the response (Brown and Roth, 1991).

In this report, isolated bovine PMN showed surface expression of TLR2 and TLR4, which is in line with human PMN (Chang et al., 2007; Hayashi et al., 2003; Sabroe et al., 2002). When exposed to different synthetic TLR ligands, bovine PMN showed a rapid increase in size and shape changes as detected by changes in the forward scatter. These changes occurred as early as after 10 sec of incubation, indicating a fast activation process in stimulated cells compared to unstimulated condition (Jain et al., 1991). Accordingly, this parameter has been interpreted as a marker of cell activation in human and bovine PMN (Lotz et al., 2004; McClenahan et al., 2000).

TLR ligands induces a slightly increase (10%) in the phagocytosis activity of bovine PMN, except for ultrapure LPS. These findings are in line with the previous observation in human PMN where the exposure to TLR ligands induces an increase in the phagocytosis of latex beads (Hayashi et al., 2003). In our experimental setup, the PMN were incubated with bioparticles derived from *S. aureus* conjugated with the pHrodo dye, and the fluorescence occurs only in an acidified medium and permits the detection of intra-phagosomal phagocytosis discarding the particles attached –but not phagocytized-to the cell membrane.

Rise in intracellular calcium concentration is an early event in the activation of bovine PMN and governs several functional responses such as ROS production, degranulation and expression changes in adhesion molecules (Burgos et al., 2011). In this context, we

intended to assess if TLRs ligands triggers an increase in the intracellular calcium concentration in bovines PMN exposed to ultrapure LPS, Pam_3CSK_4, HKLM and FSL-1. Observation of bovine PMN exposed to TLR ligands shows that only Pam_3CSK_4 10µg/ml displayed an increase in intracellular calcium influx compared with those not exposed or exposed to ultrapure LPS, HKLM and FSL-1. To our knowledge, this is the first report of increased calcium concentration in bovine PMN exposed to Pam_3CSK_4.

In the present study the exposure of bovine PMN to Pam_3CSK_4 10µg/ml resulted in an intense increase of the ROS production compared with unstimulated PMN. In addition FSL-1 induced ROS production in a later time point compared with Pam_3CSK_4. In support of this, several ligands of the TLRs have been reported as modest inductors of ROS production in human PMN and have a priming effect over the ROS production induced by fMLP (Hayashi et al., 2003; Sabroe et al., 2003). In addition, differences between species have been reported previously in terms of the agent that induces ROS production and the magnitude of this response (Brown and Roth, 1991; Styrt, 1989). In the bovine model, it was reported previously that ROS production was increased with the TLR2/TLR6/dectin-1 ligand zymosan (Nagahata et al., 2007) and LPS from *Escherichia coli* did not increase the total ROS production at a concentration of 50µg/ml (Revelo and Waldron, 2012), confirming our observations. To our knowledge this is the first report involving the activation of this parameter on bovine PMN exposed to Pam_3CSK_4. However, since TLRs were not silenced or neutralized we cannot discard that the effect elicited by Pam_3CSK_4 and FSL-1 is due to the interaction with other receptors.

MMP-9 or gelatinase B is released from granules of activated PMN and can play a role in the recruitment of PMN in the mammary gland in mastitis or in the lung of cows suffering

425 *Mannheimia haemolytica* pneumonia (Li et al., 1999; Starr et al., 2004). In the present
426 study Pam_3CSK_4 induces MMP-9 secretion compared with control condition whereas LPS,
427 HKLM nor FSL-1 not. To date, no reports on secretion of this enzyme from bovine PMN
428 induced by TLR ligands were found, but an approximation can be made with the results
429 obtained in bovine PMN incubated with zymosan, a TLR2/dectin-1 ligand that triggers a
430 dose dependent release of MMP-9 (Higuchi et al., 2007). In addition, in mice neutrophils
431 MMP-9 secretion was highly dependent of the TLR signaling adaptor protein MyD88
432 (Bradley et al., 2012) suggesting an important role of the TLR signaling in the secretion of
433 this enzyme.

434 Delayed apoptosis of PMN at the site of inflammation is an important factor that can
435 explain in part the maintenance of the inflammatory response in the tissues. In subclinical
436 mastitis, condition characterized by a persistent accumulation of PMN in milk, delayed
437 apoptosis of PMN has been described (Boutet et al., 2004). In this report, the percentage of
438 apoptotic-, alive- and dead bovine PMN exposed to ultrapure LPS, Pam_3CSK_4, HKLM and
439 FSL-1 (for 24 h) was investigated. There were no changes over these parameters suggesting
440 that the activation induced by Pam_3CSK_4 is not due to the increasing number of apoptotic
441 or dead cells. In support of this, human PMN exposed to purified LPS and Pam_3CSK_4 at
442 similar concentrations induce delayed constitutive apoptosis of cells after 4 h of treatment,
443 but this effect is not maintained when cells were incubated for 22 h (Sabroe et al., 2003). In
444 addition, a previous report on bovine PMN showed that after exposition to LPS for 20 h the
445 PMN apoptosis remained unaltered (Sohn et al., 2007), although the percentage of
446 apoptotic PMN was higher (61% average) than the value obtained in our observations
447 (23.7% average) for the unstimulated condition.

One unexpected observation was the unresponsiveness of bovine PMN to LPS in the different parameters evaluated since this molecule has been largely associated with the activation of PMN. To interpret this, at least two factors, excluding incubation time and concentrations must be taken into account. Firstly, it has been reported that different preparations of LPS can elicit different responses depending on the purification protocol used to obtain LPS. In all the experiments performed in this report an ultrapure LPS, with several enzymatic hydrolysis steps was used and it is expected that this preparation of LPS activates specifically TLR4 but not TLR2 (Hirschfeld et al., 2000; Tapping et al., 2000). The majority of reports involving activation of bovine PMN via LPS involve the utilization of LPS without further steps of purification, a factor that was described as a source of differences in the neutrophil responses (Hirschfeld et al., 2000; Sabroe et al., 2003). Secondly, LPS activation of PMN may depend on the presence of lipopolysaccharide binding protein (LBP) and the surface expression of CD 14, to form the LPS receptor complex and serum is considered an important source of these factors (Sohn et al., 2007; Soler-Rodriguez et al., 2000). In our experimental setup, isolated bovine PMN were exposed to ultrapure LPS in a medium without serum and therefore with no additional LBP. As PMN also lack CD14 expression, the observed unresponsiveness could be attributed to this. Further research is needed to confirm these assumptions.

In general, the blockage of the calcium influx results in a decreased activity of PMN, supporting the hypothesis of the potential therapeutic use of calcium entry inhibitors to treat inflammatory processes with persistent PMN infiltration in bovines (Burgos et al., 2011). 2-APB is a SOCE inhibitor that interferes with the IP3 receptor at the ER level, decreasing release of intracellular calcium from this organelle (Anderson et al., 2005; Hauser et al.,

2001). This effect causes a decrease in the calcium influx, and specifically in bovine PMN, causes the inhibition of several responses induced by PAF (Conejeros et al., 2012). MRS1845 is a dihydropiridine that inhibits voltage-dependent L-type calcium channels. In a screening of this class of molecules using HL60 cells stimulated with ATP, MRS1845 was one of the more potent inhibitors of SOCE (Harper et al., 2003) and to our knowledge this is the first report regarding the effect of MRS1845 in bovine PMN. Both SOCE inhibitors decreases the intracellular calcium concentration induced by Pam_3CSK_4 and only 2-APB decreased the ROS production but not the MMP-9 secretion indicating that the gelatinase secretion induced by Pam_3CSK_4 is independent of the SOCE signaling pathway. These results suggest that the ROS production induced by Pam_3CSK_4 is due to the release of intracellular calcium from the ER rather than the entry of extracellular calcium because MRS1845 was not able to decrease this response. This data also contributes to the evidence regarding the key role of calcium signaling as second messenger in the activation of bovine PMN by ligands from different origin.

5. Conclusions

In cattle, some of the pathologies which are characterized by the persisting presence of PMN in the injured tissue include bovine pneumonic pasteurellosis (Caswell et al., 1998) and subclinical mastitis (Boutet et al., 2004). Recognition of pathogens is critical in the initiation of inflammatory process and the activation of PMN by TLR ligands can be considered as one of the first steps of this process. The specificity of the recognition is variable between species and therefore is of interest the study of biological aspects of the innate immune system in domestic animals beyond classical models such as rodents (Werling et al., 2009). In this context this report gives useful information about the direct

activation of bovine PMN by the TLR 2/1 ligand Pam_3CSK_4. In addition, can be suggested that the SOCE inhibitor 2-APB can modulate the ROS production induced by Pam_3CSK_4 whereas the induced secretion of MMP-9 seems to be independent of the increase in intracellular calcium concentration induced by this ligand.

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664 **Figures legend**

665 **Figure 1 – Effect of synthetic TLR ligands on morphology of bovine PMN.** 250,000
666 isolated PMN were treated with the indicated concentrations of ultrapure LPS (uLPS),
667 Pam_3CSK_4, HKLM and FSL-1 and the changes in size and shape were registered by flow
668 cytometry. In the left panel dot plots of the side scatter (SSC) and forward scatter (FSC) are
669 shown. In the right panel histograms showing the shift in FSC for each treatment is shown.
670 The graphs are representative of three independent experiments.

671 **Figure 2 - TLR2 and TLR4 expression by bovine PMN.** A) Isolated bovine PMN (0.5 x
672 10^6) were incubated with antibodies against CD11b (FITC) and either TLR2 (Alexa 647) or
673 TLR4 (Alexa 647) with corresponding isotype controls. PMN were gated for CD11b
674 expression with relative expression levels for TLR2 (-----) and TLR4 (- - - -) are shown
675 relative to isotype controls (––). TLR expression levels were determined by flow cytometry
676 for 3 animals; data presented are representative with similar staining patterns observed for
677 all animals tested. Data analysis was performed using Flow Jo V10 (www.flowjo.com Tree
678 Star, Inc., USA). B) HEK cells (0.2×10^6), native and expressing bovine TLR2 or bovine
679 TLR4/MD2, were incubated with antibodies against TLR2 or TLR4 and corresponding
680 isotype controls. Relative expression levels of TLR2 or TLR4 (- - - -) are shown compared
681 to HEK cells alone (-----) and isoypte controls (––). Data analysis was performed using
682 Flow Jo V10 (www.flowjo.com Tree Star, Inc., USA).

683 **Figure 3 – Stimulation of bovine PMN with a synthetic TLR2 ligand results in**
684 **increased intracellular calcium concentration and ROS production.** A) $1x10^6$ isolated
685 bovine PMN loaded with FLUO4-AM were treated with the indicated concentrations of the
686 TLR ligands and the area under the curve (AUC) of the fluorescence emitted after 400

seconds was calculated. B) $1x10^6$ isolated bovine PMN were incubated with luminol 80µM and treated with the indicated concentrations of the TLR ligands. The chemoluminiscence was registered and the AUC was calculated. The upper X axis indicates the HKLM concentration in cells/ml and the lower X axis indicates the concentration of uLPS, Pam_3CSK_4 and FSL-1 in µg/ml. Both results were plotted in base 10 logarithmic scale. Statistical significance was determined by ANOVA and Dunnet test against the control (vehicle) condition for each ligand. *=p≤0.05, **=p≤0.01, n=3.

Figure 4 – Pam_3CSK_4-induced MMP9 secretion from bovine PMN. $1x10^6$ PMN were treated with the indicated concentrations of ultrapure LPS, Pam_3CSK_4, HKLM and FSL-1 and the supernatants were recovered and analyzed by zymography. The clear bands over blue background, indicative of gelatinolitic activity, were subject to densitometry analysis and the values were normalized to the control condition for each ligand. Bars represent the mean ± SEM of three independent experiments. Statistical significance was determined by ANOVA and Dunnet test against the control (vehicle) condition for each ligand. **=p≤0.01

Figure 5 – Pam_3CSK_4-induced MMP9 secretion partially depends on ROS production. $1x10^6$ PMN were pretreated for 15 min with the SOCE inhibitors 2-APB and MRS1845 prior to the stimulation with Pam_3CSK_4 10 µg/ml and the calcium influx (A), ROS production (B) and MMP-9 secretion was determined as described previously. Bars represent the mean ± SEM of three independent experiments. Statistical significance was determined by ANOVA and Dunnet test against the control (vehicle) condition.

Figure 6 – Stimulation with TLR ligands does not induce apoptosis in bovine PMN. 500,000 PMN were incubated with the TLR ligands in RPMI medium at the indicated

concentrations. After 24 hours, the cells were analyzed using a commercial kit for the detection of Annexin V positives (apoptotic) and Propidium iodide positive (dead) cells by flow cytometry. No changes were observed compared with the control (vehicle) condition. Bars represent the mean ± SEM of three independent experiments.

Table legend

Table 1. Incubation with TLR ligands increase the percentage of phagocytosis-positive PMN

TLR ligand	Vehicle	uLPS 1 µg/ml	Pam_3CSK_4 10 µg/ml	HKLM 10^8 cells/ml	FSL-1 1 µg/ml
% of phagocytosis-positive PMN	71.98±5.00	75.87±5.49 ^{ns}	83.85±3.31*	84.82±3.12*	84.22±2.87*

Percentages of phagocytosis-positive PMN for each condition are shown as mean ± SEM. Significance was determined by a Student *t* test comparing with the untreated condition. ns= non-significant, *=$p<0.05$ (n=6).