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Gene microarray integrated with iTRAQ-based proteomics for the discovery of NLRP3 in LPS-induced inflammatory response of bovine mammary epithelial cells

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Research Article

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Abstract

Mastitis, a major infectious disease in dairy cows, is characterized by an inflammatory response to pathogens such as *Escherichia coli* and *Staphylococcus aureus*. To better understand the immune and inflammatory response of the mammary gland, we stimulated bovine mammary gland epithelial cells (BMECs) with *E. coli*-derived lipopolysaccharide (LPS). Using transcriptomic and proteomic analyses, we identified 1019 differentially expressed genes (DEGs, fold change ≥2 and P-value < 0.05) and 340 differentially expressed proteins (DEPs, fold change ≥1.3 and P-value < 0.05), of which 536 genes and 162 proteins were upregulated and 483 genes and 178 proteins were downregulated following exposure to LPS. These differentially expressed genes were associated with 172 biological processes; 15 Gene Ontology terms associated with response to stimulus, 4 associated with immune processes, and 3 associated with inflammatory processes. The DEPs were associated with 51 biological processes; 2 Gene Ontology terms associated with response to stimulus, 1 associated with immune processes, and 2 associated with inflammatory processes. Meanwhile, several pathways involved in mammary inflammation, such as Toll-like receptor, NF-κB, and NOD-like receptor signaling pathways were also represented. *NLRP3* depletion significantly inhibited the expression of IL-1β and PTGS2 by blocking caspase-1 activity in LPS-induced BMECs. These results suggest that NLR signaling pathways works in coordination with TLR4/NF-κB signaling pathways *via* NLRP3-inflammasome activation and pro-inflammatory cytokine secretion in LPS-induced mastitis. The study highlights the function of NLRP3 in an inflammatory microenvironment, making *NLRP3* a promising therapeutic target in *Escherichia coli* mastitis.

Bovine mastitis is one of the most important world-wide infectious diseases in dairy cattle that affects both the quality and quantity of milk (Seegers *et al*., 2003), and subsequently, leads to an immeasurable economic loss to farmers (Hertl *et al*., 2014; Gomes and Henriques, 2016). This disease is characterized by an inflammatory response of the mammary tissue caused by bacterial and fungal infections. Approximately 30% of cows carry mastitis pathogens, and one third of all cows suffer from mastitis each year (Gussmann *et al*., 2019). Meanwhile, it is of a particular concern that the inevitable indiscriminate use of antibiotics in tackling cattle mastitis may worsen the antibiotic-resistance issues associated with antimicrobial therapy in humans (Im *et al*., 2014). Therefore, the development of novel therapies for mastitis is urgently needed (Fu *et al*., 2014). While vaccines are in development, the wide range of species and strains of micro-organisms causing mastitis makes this approach challenging. One alternative strategy is to improve an individual animal's ability to rid itself of the infective agents by either genetically selecting for or inducing enhanced innate immune capabilities within the population of dairy cattle. However, this strategy is critically dependent on better understanding of the innate host immune responses during mastitis (Philpott *et al*., 2001).

Bovine mammary epithelial cells (BMECs) are the main sites for milk protein synthesis and secretion. They are also a major line of defense against pathogenic bacteria and contribute significantly to the immunity of the mammary gland (Gutsmann *et al*., 2001). Once the pathogenic bacteria enter the udder, pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are produced (Corl *et al*., 2010; Kang *et al*., 2016) and local mammary inflammation events are induced (Strandberg *et al*., 2005; Im *et al*., 2014). A number of studies have shown that BMECs in culture are able to sense bacteria or bacterial products and respond by upregulating several sets of genes involved in the innate immune response (Griesbeckzilch *et al*., 2008; Ibeaghaawemu *et al*., 2008). Although a large amount of information has been gathered, the innate immune mechanisms of sensing *E. coli* in the mammary gland and subsequently inducing the immune responses are not completely understood. There were some differences in the

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mammary immune response to distinct mammary pathogenic *E. coli* strains (Blum *et al.*, 2017). Lipopolysaccharide (LPS), a structural component of the outer membrane of Gram-negative bacteria, has been identified as an important risk factor for mastitis and is often used to simulate Gram negative mastitis (Kang *et al.*, 2016). Furthermore, LPS provokes the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), which in turn activate or suppress the expression of acute phase genes in hepatocytes, vascular endothelium and other target cells (Jiang *et al.*, 2008). Although some studies have investigated the activation of LPS-induced Toll-like receptor 4 (TLR4) and NF-κB and the production of secreted cytokines IL-1β and IL-6 in BMECs (Günther *et al.*, 2016; Wang *et al.*, 2018), the NOD-like receptor (NLR) pathways involved in regulating the secretion of mature IL-1β in *E. coli* mastitis has not yet been characterized. The procession and secretion of mature IL-1β is required for the activation of NACHT, LRR and PYD domain-containing protein 3 (NLRP3) inflammasome (Wang *et al.*, 2014; Lemarchand *et al.*, 2019), and this NLRP3 inflammasome plays an important role in the development of LPS-induced mastitis (Li *et al.*, 2019).

Recently, integrating transcriptomic and quantitative proteomic analyses has been widely used to promote a better understanding of the molecular mechanisms driving biological process in cells and tissues (Wenting *et al.*, 2018). The aim of this study was (1) to elucidate a more complete understanding of molecular mechanisms underlying mammary epithelial cell inflammatory response to LPS; and (2) to identify the role of NLRP3 in LPS-induced inflammatory response. Our data may provide valuable information for uncovering the mechanism of mastitis resistance in dairy cows and assist the treatment of mastitis.

Materials and methods

The study was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University and performed in accordance with the 'Guidelines for Experimental Animals' of the Ministry of Science and Technology (Beijing, China).

Cell preparation and treatment

Mammary tissues were collected from three Holstein dairy cows that have similar age, parity, and lactation stage at a local slaughterhouse. All were at the end of lactation, two of them were 5 years old and one was 6 years old (parity = 4). They were culled due to low milk production. The overall study design is summarized in online Supplementary Fig. S1. BMECs were cultured in accordance with the established method of the Nanjing Agricultural University of Dairy Cow Science Institute (Sun *et al.*, 2015). Cells were incubated at 37 °C in 5% CO_2. Cells cultured in medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 100 U/ml of antibiotic (penicillin and streptomycin; Sigma-Aldrich, St. Louis, MO, U.S.A.) were used at passage 3 or 4 for further research. The epithelial origin and purity of the BMEC were assessed by immunofluorescence for cytokeratin 18, an epithelial cell-specific marker (online Supplementary Fig. S2).

After cells were grown to 70–80% confluence, all cells were washed twice with PBS, and then DMEM (serum free) was added to each well of culture plates. The culture plates were then divided into two groups of three plates: CON1–3 (control) and LPS1–3 (lipopolysaccharide), O55:B5, 10 μg/ml in culture medium, based on our previous study and others studies (Sun *et al.*, 2015; Qu *et al.*, 2017). After 12 h of LPS treatment, the cells were harvested and used in experiments as described in figure legends. Then, the cell culture supernatant of BMECs was collected for ELISA analysis.

RNA isolation and microarray assay

The detail steps were performed as described in the online Supplementary File Materials and methods. Total RNA was extracted from BMECs using Trizol reagent (Hoffmann-La Roche Ltd., Shanghai, China) according to the manufacturer instructions. The gene chip used in the Bovine Genome Array was generated by a service provider (SBC Co., Ltd., Shanghai). The total RNAs of the NT and LPS-treated BMECs were individually hybridized with gene chips. Affymetrix Micro Array Suite 5.0-Specific Terms GCOS v1.4 was used for quantitative analysis of the hybridization gene expression levels; those with ≥ 2-fold (P-value < 0.05) difference between the groups were checked and further analyzed (Affymetrix microarray analysis followed by RMA normalization method). The Molecule Annotation System (http://david.abcc.ncifcrf.gov/) was used to analyze the differentially expressed genes, using the Kyoto encyclopedia of genes and genomes (KEGG) public pathway resource and the gene ontology (GO) consortium.

Protein extraction, quantification and digestion

The detail steps were performed as described in the online Supplementary Materials and Methods. After protein extraction and protein digestion, equal volumes of 0.1% formamide (FA) was added to acidify the solution. Peptides were purified on a Strata-X C18 pillar three times, washed with 0.1% FA + 5% acetonitrile (ACN) twice, and eluted with 1 ml 0.1% FA + 80% ACN. Eluted peptides were dried with a vacuum concentration meter. The dried peptide powder was re-dissolved in 20 μl of 0.5 mol/l triethylamine borane (TEAB) for peptide labeling.

iTRAQ labeling and fractionation

Samples were labeled with iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex UK Ltd., Warrington, UK). Control groups were labeled as iTRAQ channels 113–115 and LPS-treated BMECs were labeled as iTRAQ channels 116–118. All of the labeled samples were mixed in equal amounts. Next, the labeled samples were fractionated using high performance liquid chromatography (HPLC) (Thermo DINOEX Ultimate 3000 BioRS, THERMO FISHER, Berlin, Germany) using a Durashell C18 (5um, 100 Å, 4.6 × 250 mm). Finally, 12 fractions were collected.

LC-MS/MS and data analysis

Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis was performed on an AB Sciex nano LC-MS/MS (Triple TOF 5600 plus) system. The detailed steps were performed as described in the online Supplementary Materials and Methods

The original MS/MS file data were submitted to Protein Pilot Software v4.5 (Applied Biosystems, Foster City, CA, USA) for data analysis. For protein identification, the Paragon algorithm integrated into Protein Pilot was employed against the SwissProt

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bovine database from Uniprot website (http://www.uniprot.org) using Mascot software version 2.3.02 (Matrix Science, London, UK). The following filter was used in this study: 1% false positive rate at the protein level and two unique peptides for each protein. After filtering the results, the peptide abundances in the different reporter ion channels of MS/MS scan were normalized. For DEPs determination, fold changes were calculated as the average comparison pairs among biological replicates. Proteins with a fold change larger than 1.3 and *P*-value < 0.05 were considered to be significantly differentially expressed. The Molecule Annotation System (http://david.abcc.ncifcrf.gov/) was used to analyze the differentially expressed proteins. The GO terms of biological process in DAVID (http://david.abcc.ncifcrf.gov/) were employed to categorize enriched biological themes in differentially expressed protein lists.

Cytokine and apoptosis assays

The cell supernatant was used to evaluate the levels of cytokines of TNF-α (the intra- and inter- assay CV were 3.64 and 4.39%), IL-1β (the intra- and inter- assay CV were 2.45 and 2.66%) and IL-6 (the intra- and inter- assay CV were 4.21 and 3.86%) with the ELISA kits under the instructions of the manufacturer (BioLegend, Inc., Camino Santa Fe, Suite E, San Diego, CA, USA).The level of apoptosis was detected using the Annexin V-FITC kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, after the indicated treatments, cells were collected and stained with Annexin V-FITC following the manufacturer's instructions. The stained cells were then counted by using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The equation (number of cells in the upper right quadrant + cells in the lower right quadrant)/(total cell number) was used to calculate the rate of apoptosis.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed to confirm the microarray results. Total RNA was extracted from mixed BMECs as described above and was reverse transcribed using a reverse transcription level kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The GAPDH *rRNA* gene was used as an invariant control. Primers were designed using Primer Premier 5.0 and are shown in online Supplementary Table S1. RT-PCR was performed with SYBR®Premix Ex Taq™ (Takara). The reaction solution was prepared on ice and comprised 10 µl of 2 × SYBR®Premix Ex Taq™, 0.8 µl PCR forward primer (10 µM), 0.8 µl PCR reverse primer (10 µM), 0.4 ml 50 ROX reference dye, 2 µl cDNA (100 ng/µl), and distilled H_2O to a final volume of 20 µl. The reaction mixtures were incubated in a 96-well plate at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. All target genes were normalized to the endogenous reference gene GAPDH by employing an optimized comparative Ct $^{(2^{-\Delta\Delta Ct})}$ value method, where ΔΔCt = ΔCt target − ΔCt GAPDH.

Protein isolation and western blotting

Total protein from BMECs (all three cows) was isolated using a tissue protein extraction kit (Bio-Rad, Shanghai) and quantified using a Bradford Assay Kit (Sangon Biotech Co., Ltd, Shanghai, China). For western blotting, the extracted materials (30–50 µg/lane) were subjected to gel electrophoresis (8% SDS-PAGE) followed by electrotransfer to a polyvinylidene fluoride membrane. The membrane was then blocked with 5% freshly-prepared milk/Tris-buffered saline containing 20% Tween (TBST) for 2 h at RT and then incubated overnight at 4 °C with primary antibodies {rabbit anti-rat NLRP3 (1:1000, Novus), Pro-caspase-1 (1:1000, Novus), IL-1β (1:1000, Abcom, Cambridge, USA), or COX-2 (1:1000, Abcom, Cambridge, USA)}. After three washes in TBST, the membrane was incubated with peroxidase-conjugated secondary antibody (GGHL-15P anti-IgG, 1:1000 dilution; Santa Cruz Biotechnology, CA, USA) in blocking solution for 2 h at RT. After a final wash in TBST, the presence of the secondary antibodies was visualized using ECL reagent (Amersham Life Science, Arlington Heights, MA, USA). In all analyses, probing with anti-β-actin was used to monitor sample loading.

siRNA interference

The BMECs were seeded in a six-well-plate and cultured for 24 h until they reached 50–60% confluency. The cells were transfected with 20 µM of either experimental siRNA oligos or a non-targeting control with Lipofectamine®2000 (Invitrogen, Carlsbad, CA, USA). The knockdown efficiency was determined using qRT-PCR and western blotting. The NLRP3 and negative control (NC) siRNA sequences were synthesized by Shanghai GenePharma (Shanghai, People's Republic of China). The sequences of siRNA and knockdown efficiency are shown in online Supplementary Table S2 and Fig. S3.

Statistical analysis

All data were obtained from each single independent experiment carried out in triplicate. Main and interactive effects were analyzed by one-way analysis of variance (ANOVA) using SPSS16.0 software (Chicago, IL). *P*-value < 0.05 were considered statistically significant.

Results

Differentially-expressed genes and proteins in LPS-induced BMECs

Using transcriptomics and proteomics analysis, we identified 1019 DEGs (fold change ≥2 and *P*-value < 0.05) and 340 DEPs (fold change ≥1.3 and *P*-value < 0.05), of which 536 genes and 162 proteins were upregulated and 483 genes and 178 proteins were downregulated following exposure to LPS (online Supplementary Full Data Set).

GO analyses of DEGs and DEPs

To clarify the different biological patterns of the two groups, GO analysis of DEGs and DEPs were conducted. The GO results showed that the DEGs were associated with 172 biological processes, which consisted of 15 GO terms associated with response to stimulus, 4 associated with defense processes, and 3 associated with inflammatory process. The DEPs were associated with 51 biological processes, which consisted of 2 GO terms associated with response to stimulus, 1 associated with defense processes and 2 associated with inflammatory processes. The full list is provided in Supplementary Table S3.

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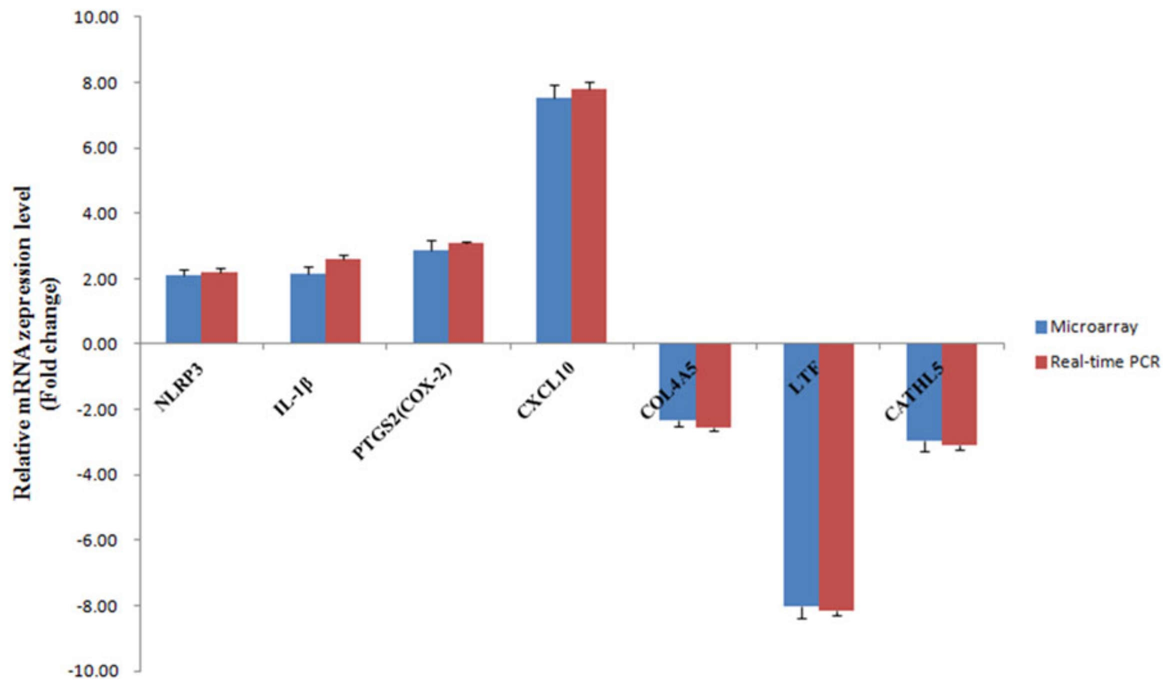

Fig. 1. Comparison of RT-PCR findings to microarray results by fold-change of 7 select genes. The fold-changes upon LPS stimulation for 7 representative BMEC DEGs are shown, as determined by microarray analysis (blue) and verified by RT-PCR (red). The real-time data represent averages of triplicates and were normalized to *GAPDH*.

Validation of differentially expressed genes and proteins related to stimulus, defense, and inflammatory responses

To characterize DEGs and DEPs related to stimulus, defense, and inflammatory responses in BMEC that were affected by LPS, the upregulated expressions of *PTGS2* and *NLRP3* genes at both transcript and protein levels were screened. GO analysis showed that *PTGS2* and *NLRP3* were involved in acute inflammatory responses (online Supplementary Full Data Set). To confirm the microarray results for the DEGs, real-time RT-PCR was performed for 7 genes, including *NLRP3*, *IL-1*, *CXCL10*, *PTGS2 (COX-2)*, *COL4A5*, *CATHL5*, and *LTF* (Fig. 1). As shown in Fig. 1, *NLRP3*, *IL-1*, *PTGS2(COX-2)*, and *CXCL10* were upregulated, while *COL4A5*, *CATHL5*, and *LTF* were downregulated, as determined by both the microarray assays and real-time RT-PCR. To confirm the iTRAQ results for the DEPs, western blotting was performed for inflammation-associated protein-NLRP3 and PTGS2 (COX-2) (Fig. 2). The expression of NLRP3 and PTGS2 (COX-2) were upregulated.

KEGG pathway analyses of DEGs and DEPs

KEGG analysis showed that the differentially expressed mRNAs were mainly involved in pathways associated with Chemokine, TNF, TLR, NF-κB, and NLR signaling pathways (Fig. 3a). Protein expression changes that reached statistical significance were analyzed in KEGG. Differentially expressed proteins were associated with the following signaling pathways: ECM-receptor interaction, focal adhesion, ribosome, and the PI3K-Akt signaling pathway (Fig. 3b).

LPS induced the expression of inflammatory cytokines and apoptosis of BMECs

To investigate the inflammatory effects of LPS, LPS-induced inflammatory cytokine production and apoptosis were measured in this study. As shown in Fig. 4a, compared with the control group, levels of TNF-α, IL-1β, and IL-6 markedly increased after LPS stimulation. As shown in Fig. 4b, compared with the control group, apoptosis rate of BMECs markedly increased after LPS stimulation ($P < 0.05$).

Confirmation of the important role of NLRP3 in inflammatory responses

To investigate the important role of NLRP3 in inflammatory responses, we used three pairs of NLRP3 siRNAs to knock down gene expression. The mRNA expression level of NLRP3 was examined by qRT-PCR after the transfection of BMECs with NLRP3 siRNA. Hence, we chose siRNA-3 (siNLRP3) for the subsequent experiment (online Supplementary Table S2 lists all the siRNA sequences and screening results). The siControl cells and siNLRP3 cells were simultaneously treated with LPS. The expression of NLRP3, pro-caspase-1, IL-1β, and PTGS2 was analyzed by western blotting. As shown, siRNA against NLRP3 successfully down-regulated the protein levels of NLRP3 in LPS-treated BMECs (Fig. 5). Also, the siRNA of NLRP3 diminished the expression of pro-caspase-1, IL-1β and PTG2 in LPS-stimulated BMECs, indicating that NLRP3 may play an important role in the inflammatory response of BMECs.

Discussion

In this study, we performed a comprehensive evaluation of expression profile in the LPS-induced BMECs by microarray and iTRAQ analysis, providing new data on the in vitro events occurring in the mammary epithelium during persistent infection. Subsequently, GO enrichment and pathway analyses of DEGs and DEPs in combination with RT-PCR, western blotting and ELISA detection for genes focused on the consistent results

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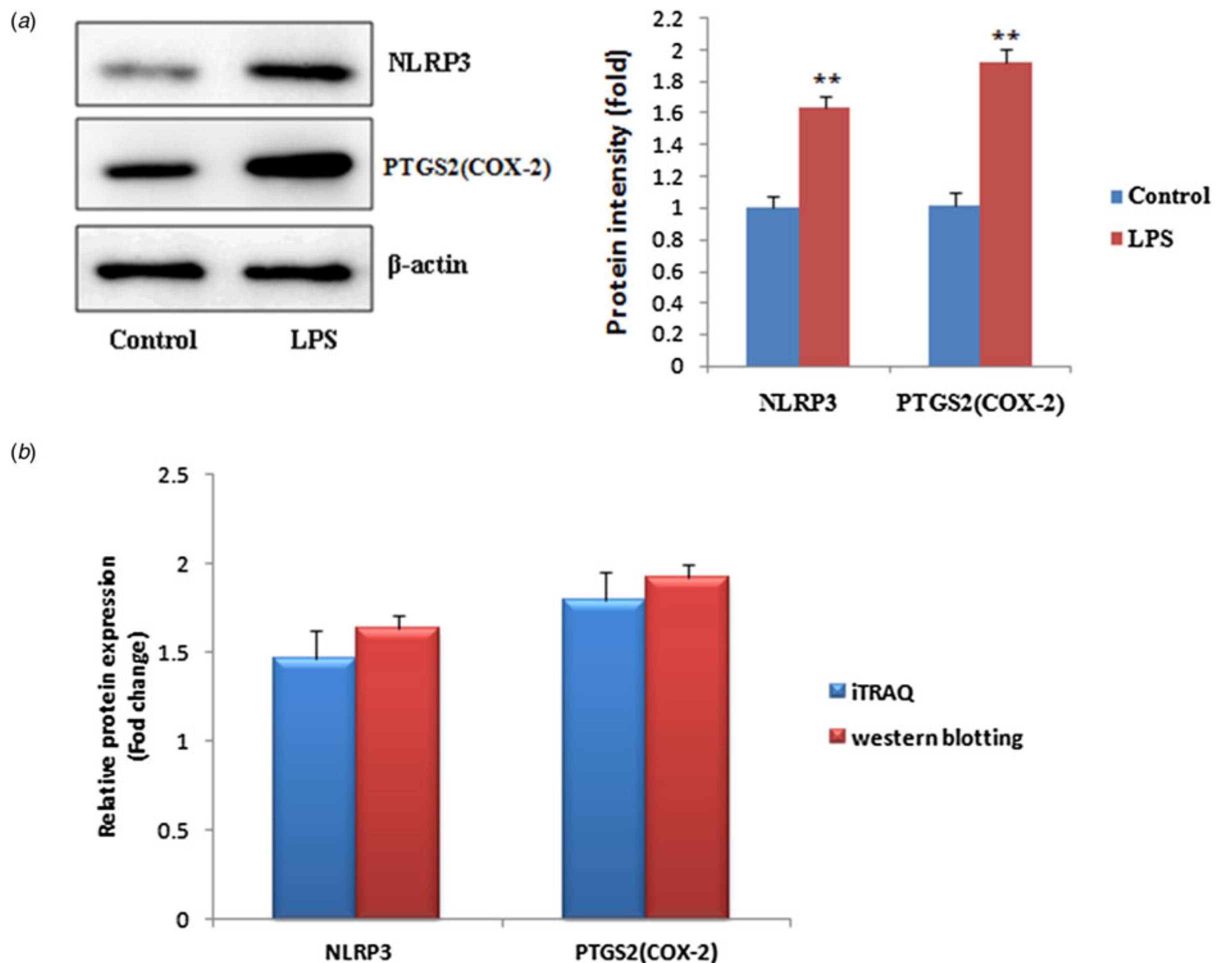

Fig. 2. Comparison of western blotting findings to iTRAQ results by fold-change of 2 select proteins. (a) The protein expression of *NLRP3* and *PTSG2 (COX-2)* were analyzed by western blotting with specific antibodies. β-actin was used as a control. The values presented are the means ± SEM of three independent experiments. (b) The fold-changes upon LPS stimulation for 2 representative BMEC DEPs are shown, as determined by iTRAQ analysis (blue) and verified by western blotting (red). $^*P < 0.05$ and $^{**}P < 0.01$ are significantly different from the control group.

involved in the inflammatory response for mastitis infection. To date, the mechanism of LPS-induced epithelial injury has mainly concentrated on TLR-4 pathway (Yang *et al*., 2008, 2016; Porcherie *et al*., 2012), while the crosstalk between TLRs and NLRs is rarely mentioned. As many studies on LPS-induced inflammatory responses have focused on TLR-4/NF-κB pathways, we verified the NLRs signaling pathways involved in the LPS-induced inflammatory response and clarified the role of the NLRP3 inflammasome in BMECs.

Our GO analysis showed that the DEGs and DEPs are involved in many inflammatory responses processes (online Supplementary Table S3). Compared to the KEGG analysis of DEGs, which revealed the involvement of many inflammatory pathways, the analysis of DEPs were mainly related to ECM-receptor interaction and the PI3K-Akt signaling pathway, which plays an important role in cell metabolism, growth, proliferation, and survival, as well as synthesis and metabolic pathways (Fig. 3). These differences between DEGs and DEPs may be due to inconsistent expression level at the mRNA and protein levels, but the exact mechanism still needs further research.

In the present study, we found many DEGs involved in multiple inflammatory response signaling pathways, such as toll-like receptors (TLRs), NF-κB, and NLRs signaling pathways. During microbial infection, crosstalk between TLRs and NLRs has been reported (Becker and O'Neill, 2007). This regulates inflammation processes and establishes clear interplay, which leads to the secretion of mature interleukin-1β (IL-1β) during microbial infection or injury (Yang *et al*., 2016). The NLRs family contains more than 20 members in mammals, and the activated member can form multiprotein complexes (called inflammasomes). These inflammasomes activate caspase-1 and induce the secretion of pro-inflammatory IL-1β and IL-18. One of the best-described is NLRP3, which acts as a sensor of metabolic stress and plays a key role in inflammation (Franchi *et al*., 2012). NLRP3 inflammasome plays an important role in the development of LPS-induced mastitis (Qu *et al*., 2017). The procession and secretion of IL-1β is required for the activation of NLRP3 inflammasome (Wang, *et al*., 2014; Lemarchand *et al*., 2019). Once activated, NLRP3 interacts with apoptosis-associated speck-like protein containing a CARD (ASC), then leads to mutual recognition of NLRP3 and pro-caspase-1, followed by activating caspase-1 (Mariathasan and Monack, 2007). The activation of caspase-1 is responsible for the secretion of the mature IL-1β (Guo *et al*., 2015; Ozaki, *et al*., 2015; Jo *et al*., 2016). NLRP3 was not only important for the assembly of the inflammasome and maturation of IL-1β, but also caused cellular changes that affected host-bacteria

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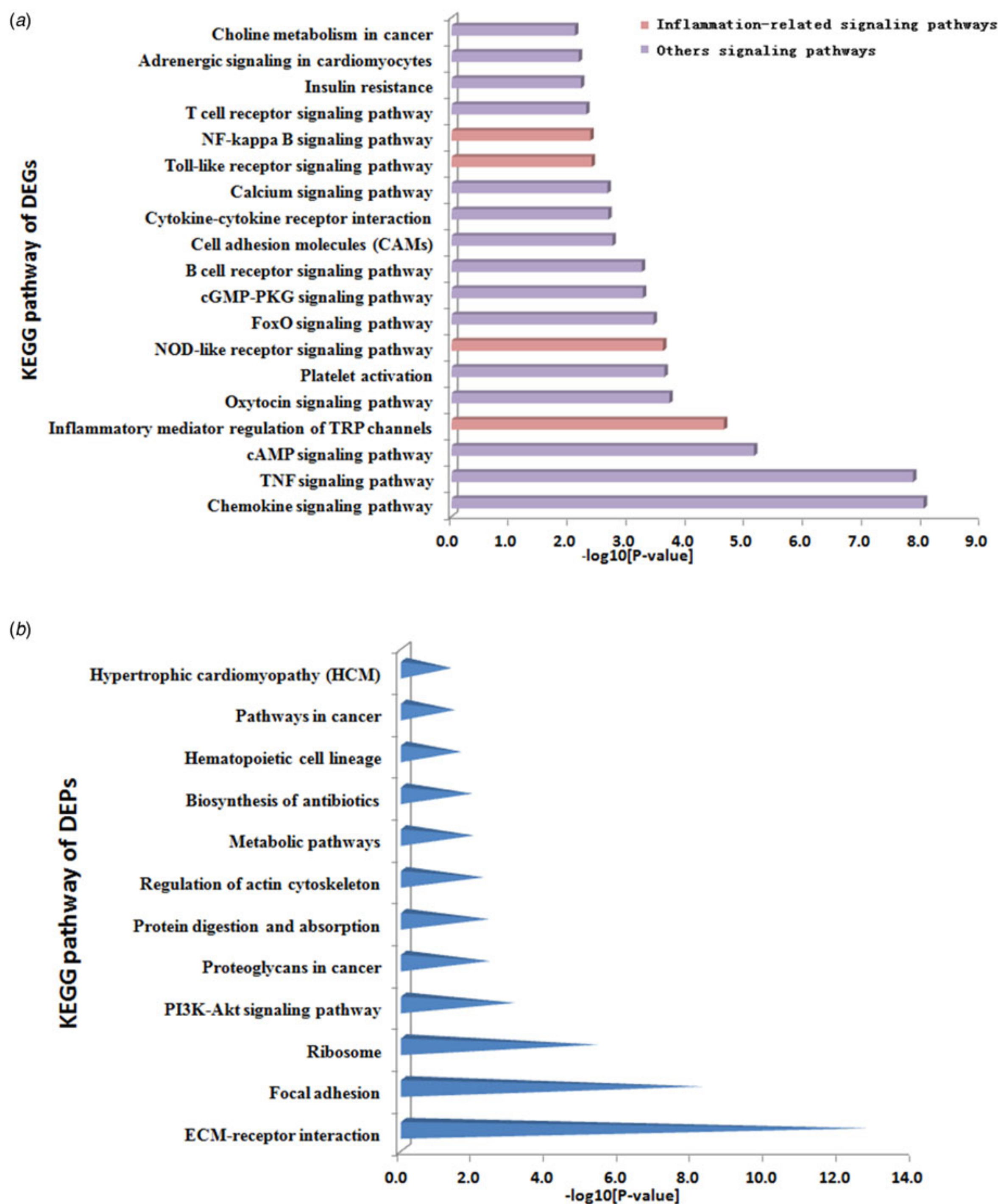

Fig. 3. KEGG analysis of the differentially expressed genes ($P < 0.01$, gene number >10) and proteins ($P < 0.05$, gene number >5). (a) The KEGG analysis results showed that the most enriched pathways included chemokine signaling pathway, TNF signaling pathway, NOD-like receptor signaling pathway, Toll-like receptor signaling pathway, NF-κB signaling pathway, Inflammatory mediator regulation of TRP channels, etc. (b) The KEGG analysis results showed that the most enriched pathways included ECM-receptor interaction, Focal adhesion, Ribosome, PI3K-Akt signaling pathway, etc.

interactions in Bladder Epithelial Cells (Demirel *et al.*, 2018) and BMECs (Li *et al.*, 2019). In the present study, we found that *NLRP3* gene was upregulated at both transcript and protein levels, and knockdown of *NLRP3* expression inhibited the expression of pro-caspase-1, IL-1β, and PTGS (COX-2). This finding is consistent with previous studies, in which the authors found that both Caspase-1 and IL-1β production was decreased after knocking down NLRP3 (Liu *et al.*, 2017). Furthermore, the expression of IL-1β was subject to partial inhibition by the knockdown of *NLRP3* gene, which means that IL-1β may derive from both of two distinct inflammatory pathways, TLR/NF-κB and NOD-like/NLRP3. This possibility is shown schematically in Fig. 6.

Interleukin-1β is also involved in the synthesis of prostaglandins (PGs) and lipid eicosanoids through cyclooxygenase enzyme

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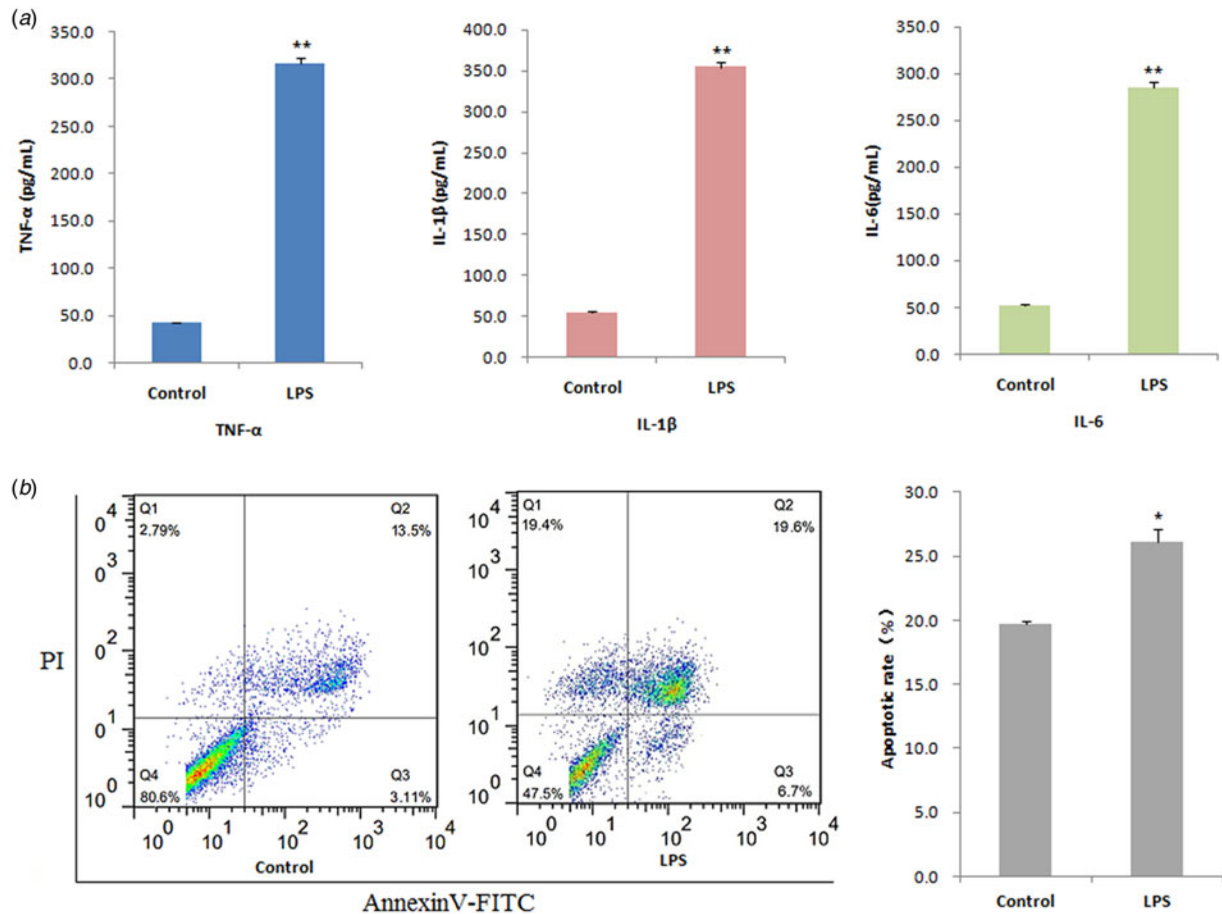

Fig. 4. Effects of LPS on proinflammatory cytokines production and apoptosis. (a)The levels of TNF-α, IL-1β and IL-6 markedly increased. (b) After BMEC were treated with 10 μg/ml of LPS for 12 h, the relative number of cells undergoing apoptosis was determined by flow cytometry after annexin V/propidium iodide (PI) staining. $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the control group.

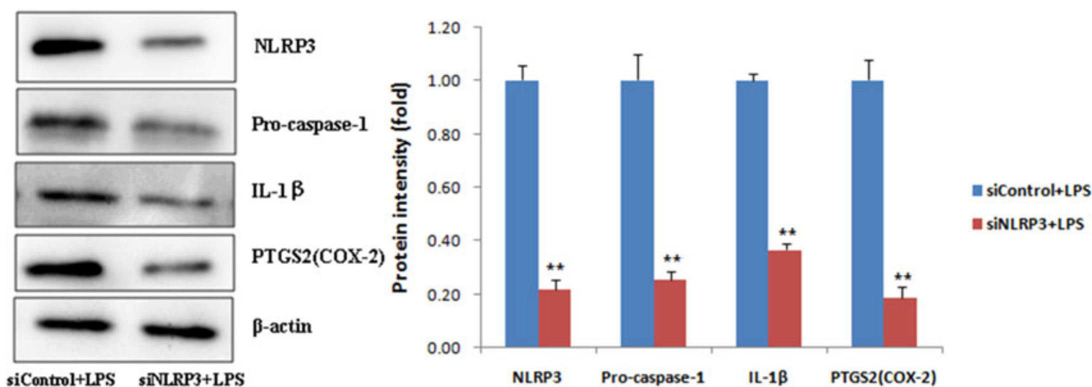

Fig. 5. Effects of silencing NLRP3 on proinflammatory cytokines expression in BMECs. Cells with stable silencing of NLRP3 with siRNA (siControl and siNLRP3) and treatment with LPS. $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the control group.

(COX) activity (Shaftel *et al.*, 2007). COX is a key enzyme that presents two isoforms: the constitutive enzyme COX-1, which is present in most cells, and COX-2, which is induced in response to proinflammatory stimuli, growth factors, infections, and other harmful stimuli (Abdallah *et al.*, 2017). COX-2 is an inflammatory mediator that converts arachidonic acid into prostaglandin E, a protein involved in inflammatory responses. COX-2 activation is a reliable end-point in cells suffering from inflammation. Previous studies have shown that there is increased expression of COX-2 in bovine mastitis (Corl *et al.*, 2010; Kang *et al.*, 2016). In the present study, LPS induced the expression of COX-2 at both transcript and protein levels. However, the molecular mechanisms involved in these processes need to be further elucidated.

In conclusion, our results demonstrate that LPS-induced mammary inflammation involves several signaling pathways

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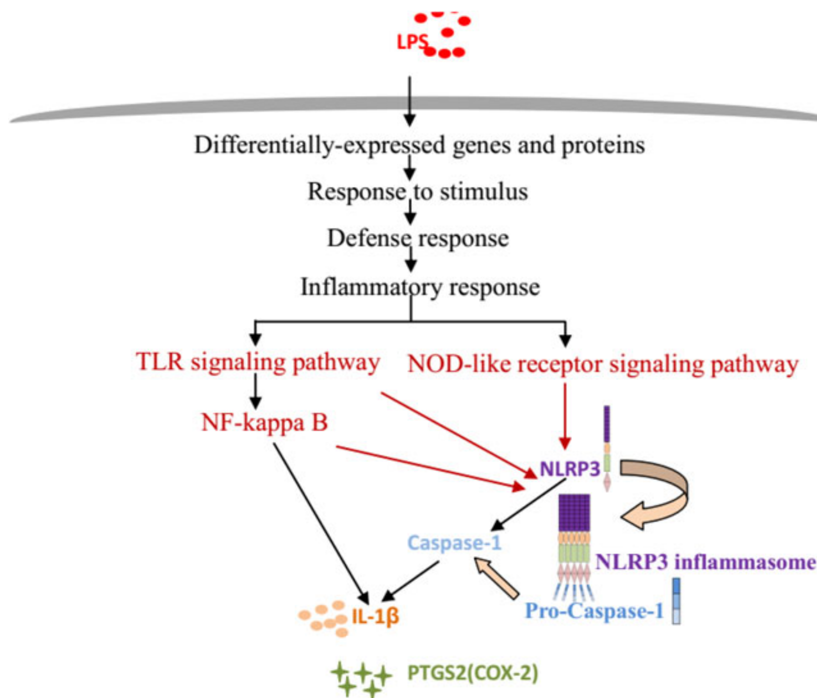

Fig. 6. Proposed mechanisms of LPS-induced inflammatory response in bovine mammary epithelial cells. Differently expression genes and proteins were involved in several signaling pathways associated with inflammatory signaling pathways. Depletion of NLRP3 partially inhibited inflammatory cytokine secretion. Consistent with previous studies, we found that NLRP3 depletion-inhibited inflammatory cytokine secretion was mediated by Pro-caspase-1. Meanwhile, the increased expression of IL-1β inhibited the PTGS3(COX-2) expression activity. These results suggested that NLRs signaling pathways works in coordination with TLR4/NF-κB signaling pathways *via* NLRP3-inflammasome activation and pro-inflammatory cytokine secretion in LPS-induced mastitis.

including the TLR4, NF-κB, and NLRs signaling pathways. NLRs signaling pathways works in coordination with TLR4/NF-κB signaling pathways *via* NLRP3-inflammasome activation and proinflammatory cytokine secretion in LPS-induced mastitis. The findings indicate the essential role of NLRP3 in bovine mastitis and may provide an effective therapeutic approach for *E-coli* mastitis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000761

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Conflict of interest. The authors declare that they have no competing interests.

References

Abdallah MS, Crj K, Stephan JS, Khalil PA, Mroueh M, Eid AA and Faour WH (2017) Transforming growth factor-β1 and phosphatases modulate cox-2 protein expression and tau phosphorylation in cultured immortalized podocytes. *Inflammation Research* **67**, 1–11.

Becker CE and O'Neill LAJ (2007) Inflammasomes in inflammatory disorders: the role of TLRs and their interactions with NLRs. *Seminars in Immunopathology* **29**, 239–248.

Blum SE, Heller ED, Jacoby S, Krifucks O and Leitner G (2017) Comparison of the immune responses associated with experimental bovine mastitis caused by different strains of *Escherichia coli*. *Journal of Dairy Research* **84**, 190.

Corl C, Robinson H, Contreras G, Holcombe S, Cook V and Sordillo L (2010) Ethyl pyruvate diminishes the endotoxin-induced inflammatory response of bovine mammary endothelial cells. *Journal of Dairy Science* **93**, 5188–5199.

Demirel I, Persson A, Brauner A, Särndahl E, Kruse R and Persson K (2018) Activation of the NLRP3 inflammasome pathway by uropathogenic *Escherichia coli* is virulence factor-dependent and influences colonization of bladder epithelial cells. *Frontiers in Cellular and Infection Microbiology* **8**, 81 doi: 10.3389/fcimb.2018.00081.

Franchi L, Muñoz-Planillo R and Núñez G (2012) Sensing and reacting to microbes through the inflammasomes. *Nature Immunology* **13**, 325–332.

Fu Y, Gao R, Cao Y, Guo M, Wei Z, Zhou E, Li Y, Yao M, Yang Z and Zhang N (2014) Curcumin attenuates inflammatory responses by suppressing TLR4-mediated NF-κB signaling pathway in lipopolysaccharide-induced mastitis in mice. *International Immunopharmacology* **20**, 54–58.

Gomes F and Henriques M (2016) Control of bovine mastitis: old and recent therapeutic approaches. *Current Microbiology* **72**, 377–382.

Griesbeckzilch B, Meyer HH, Kühn CH, Schwerin M and Wellnitz O (2008) *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *Journal of Dairy Science* **91**, 2215–2224.

Guo H, Callaway JB and Ting JP (2015) Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nature Medicine* **21**, 677–687.

Gussmann M, Steeneveld W, Kirkeby C, Hogeveen H, Farre M and Halasa T (2019) Economic and epidemiological impact of different intervention strategies for subclinical and clinical mastitis. *Preventive Veterinary Medicine* **166**, 78–85.

Gutsmann T, Müller M, Carroll SF, Mackenzie RC, Wiese A and Seydel U (2001) Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infection and Immunity* **69**, 6942.

Günther J, Petzl W, Zerbe H, Schuberth HJ and Seyfert HM (2016) TLR ligands, but not modulators of histone modifiers, can induce the complex immune response pattern of endotoxin tolerance in mammary epithelial cells. *Innate Immunity* **23**, 155–164.

Hertl JA, Schukken YH, Welcome FL, Tauer LW and Gröhn YT (2014) Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows. *Journal of Dairy Science* **97**, 1465–1480.

Ibeaghaawemu EM, Lee JW, Ibeagha AE, Bannerman DD, Paape MJ and Zhao X (2008) Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. *Veterinary Research* **39**, 11.

Im J, Lee T, Jeon JH, Baik JE, Kim KW, Kang SS, Yun CH, Kim H and Han SH (2014) Gene expression profiling of bovine mammary gland epithelial cells stimulated with lipoteichoic acid plus peptidoglycan from *Staphylococcus aureus*. *International Immunopharmacology* **21**, 231–240.

https://doi.org/10.1017/S0022029919000761 Published online by Cambridge University Press

Jiang L, Sørensen P, Røntved C, Vels L and Ingvartsen KL (2008) Gene expression profiling of liver from dairy cows treated intra-mammary with lipopolysaccharide. *BMC Genomics* **9**, 443–443.

Jo EK, Kim JK, Shin DM and Sasakawa C (2016) Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular and Molecular Immunology* **13**, 148–159.

Kang S, Lee JS, Lee HC, Petriello MC, Kim BY, Do JT, Lim DS, Lee HG and Han SG (2016) Phytoncide extracted from pinecone decreases LPS induced inflammatory responses in bovine mammary epithelial cells. *Journal of Microbiology and Biotechnology* **26**, 579–587.

Lemarchand E, Barrington J, Chenery A, Haley M, Coutts G, Allen JE, Allan SM and Brough D (2019) Extent of ischemic brain injury after thrombotic stroke is independent of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome. *Stroke* **50**, 1232–1239.

Li CM, Wang XL, Kuang MQ, Li L, Wang YR, Yang FX and Wang GL (2019) UFL1 modulates NLRP3 inflammasome activation and protects against pyroptosis in LPS-stimulated bovine mammary epithelial cells. *Molecular Immunology* **112**, 1–9.

Liu YG, Chen JK, Zhang ZT, Ma XJ, Chen YC, Du XM, Liu H, Zong Y and Lu GC (2017) NLRP3 inflammasome activation mediates radiation-induced pyroptosis in bone marrow-derived macrophages. *Cell Death & Disease* **8**, e2579.

Mariathasan S and Monack DM (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nature Reviews Immunology* **7**, 31–40.

Ozaki E, Campbell M and Doyle SL (2015) Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *Journal of Inflammation Research* **8**, 15–27.

Philpott DJ, Girardin SE and Sansonetti PJ (2001) Innate immune responses of epithelial cells following infection with bacterial pathogens. *Current Opinion in Immunology* **13**, 410–416.

Porcherie A, Cunha P, Trotereau A, Roussel P, Gilbert FB, Rainard P, Gilbert FB, Rainard P and Germon P (2012) Repertoire of *Escherichia coli* agonists sensed by innate immunity receptors of the bovine udder and mammary epithelial cells. *Veterinary Research* **43**, 14.

Qu S, Wang W, Li D, Li S, Zhang L, Fu Y and Zhang N (2017) Mangiferin inhibits mastitis induced by LPS *via* suppressing NF-κB and NLRP3 signaling pathways. *International Immunopharmacology* **43**, 85–90.

Seegers H, Fourichon C and Beaudeau F (2003) Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* **34**, 475–491.

Shaftel SS, Carlson TJ, Olschowka JA, Kyrkanides S, Matousek SB and O'Banion MK (2007) Chronic interleukin-1beta expression in mouse brain leads to leukocyte infiltration and neutrophil-independent blood brain barrier permeability without overt neurodegeneration. *Journal of Neuroscience* **27**, 9301–9309.

Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M and Tellam R (2005) Lipopolysaccharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. *Cytokine* **31**, 72–86.

Sun Y, Li L, Wu J, Yu P, Li C, Tang J, Li XJ, Huang S and Wang GL (2015) Bovine recombinant lipopolysaccharide binding protein (BRLBP) regulated apoptosis and inflammation response in lipopolysaccharide-challenged bovine mammary epithelial cells (BMEC). *Molecular Immunology* **65**, 205–214.

Wang X, Feng S, Ding N, He Y, Li C, Li M, Ding X, Ding H, Li J, Wu J and Li Y (2018) Anti-inflammatory effects of berberine hydrochloride in an LPS-induced murine model of mastitis. *Evidence-Based Complementary and Alternative Medicine*, **2018**, 1–9.

Wang YJ, Gong GQ, Chen S, Xiong LY, Zhou XX, Huang X and Kong WJ (2014) NLRP3 inflammasome sequential changes in *Staphylococcus aureus*-induced mouse model of acute rhinosinusitis. *International Journal of Molecular Sciences* **15**, 15806–15820.

Wenting D, Quanjuan W, Fengqi Z, Jianxin L and Hongyun L (2018) Understanding the regulatory mechanisms of milk production using integrative transcriptomic and proteomic analyses: improving inefficient utilization of crop by-products as forage in dairy industry. *BMC Genomics* **19**, 403.

Yang W, Zerbe H, Petzl W, Brunner RM, Günther J, Draing C, von Aulock S, Schuberth HJ and Seyfert HM (2008) Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-kappaB in mammary epithelial cells and to quickly induce TNFalpha and interleukin-8 (cxcl8) expression in the udder. *Molecular Immunology* **45**, 1385–1397.

Yang B, Zhou Z, Li X and Niu J (2016) The effect of lysophosphatidic acid on toll-like receptor 4 expression and the nuclear factor-κB signaling pathway in THP-1 cells. *Molecular and Cellular Biochemistry* **422**, 1–9.

https://doi.org/10.1017/S0022029919000761 Published online by Cambridge University Press