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UFL1 modulates NLRP3 inflammasome activation and protects against pyroptosis in LPS-stimulated bovine mammary epithelial cells

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ABSTRACT

UFL1 was identified as a key regulator of cellular stress, which was found to possess anti-inflammatory and cytoprotection effect in LPS-stimulated bovine mammary epithelial cells in our previous study. The NLRP3 inflammasome, which responds to various pathogenic microorganisms and sterile stressors, is involved in multiple inflammatory diseases. However, the specific effects of UFL1 on NLRP3 inflammasome activation remain elusive. Here we investigated the role of UFL1, with a focus on NLRP3 inflammasome activation and the regulation of pyroptosis in LPS-stimulated BMECs. In this study, we observed an elevating NLRP3, Caspase-1 activation and IL-1β secretion in mammary tissue of cows with mastitis and LPS-stimulated BMECs, and the experimental results here demonstrated that UFL1 depletion aggravated the LPS-induced NLRP3, Caspase-1 and IL-1β expression. Overexpression of UFL1 significantly suppressed the expression of NLRP3, Caspase-1 and IL-1β in BMECs. In addition, the suppression of NLRP3 inflammasome activation by UFL1 was partly mediated through the regulation of NF-κB signaling and ROS production. Furthermore, UFL1 overexpression could alleviate NLRP3 inflammasome activation-mediated pyroptosis in LPS-stimulated BMECs. These findings indicate that UFL1 can moudulate NLRP3 inflammasome activation and serve as effective strategies to diminish cell damage in inflammatory response by targeting NLRP3 inflammasome activation.

1. Introduction

Mastitis is an inflammation of the mammary gland, which is most often caused by the infection of mammary gland by various micro-organisms, including gram-negative bacteria, Escherichiacoli. Bovine mammary epithelial cell (BMEC), the predominant cell type in the mammary gland, can bind gram-negative bacterial lipopolysaccharide (LPS) to pathogen-associated molecular pattern molecules via the activation of various pattern recognition receptors (PRRs), e.g. toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs), leading to a sequential cascade of different transcriptional regulatory events, resulting in bovine mastitis (Fukata et al., 2009; Kolattukudy and Niu, 2012; Porcherie et al., 2012).

The inflammasomes are multi-protein cytoplasmic complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and Caspase 1, which intersect with a wide variety of immune and cell death pathways (Latz et al., 2013a; Martinon et al., 2002). The NLRP3 (NOD-like receptor family, Pyrindomain-containing 3) inflammasome, which formed by NLRP3, adapter protein ASC and Caspase 1, is an essential mediator of immune responses and can be activated by various stimuli, including pathogens, environment stress, tissue damage and metabolic dysregulation (Broz and Dixit, 2016; Mao et al., 2013; Tang et al., 2017). Upon stimulation, the protein complex is assembled to recruit and cleave the precursor of Caspase-1 (pro-Caspase-1, p45) to its active form (Croker et al., 2014; Guo et al., 2015). Active Caspase-1 then results in the cleavage and secretion of biologically active forms of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 (Cookson and Brennan, 2001; Ghayur et al., 1997; Kostura et al., 1989). Furthermore, studies show that activation of Caspase 1 by NLRP3 inflammasome regulates the cell death process known as pyroptosis in response to infections and cellular stress (Hu et al., 2017; Li et al., 2016; Santoni et al., 2015). Pyroptosis, a proinflammatory form of regulated cell death distinct from apoptosis, is emerging as a general innate immune effector mechanism in mammals (Jorgensen and Miao, 2015). It is a Caspase-1 dependent process by definition and differs from other types of cell death. Morphologically, cell death by pyroptosis is accompanied by plasma membrane rupture, cytoplasmic swelling and osmotic lysis, which results in DNA cleavage and the release of pro-inflammatory cellular contents (Brodsky and Medzhitov, 2011).

UFL1(also known as KIAA0776, NLBP and Maxer), which was originally characterized as an UFM1 E3 ligase that played essential roles in protein ufmylation, is a novel protein with multiple function (Zhang

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et al., 2015). It has recently been identified by independent studies as an important regulator of NF-κB signaling and cellular stress response. In the previous study, we have found that UFL1 can engage TLR4/NF-κB pathway and efficiently mediates LPS-induced ER stress and autophagy, thereby alleviates inflammatory response and cell damage. However, whether UFL1 could mediate LPS-induced NLRP3 activation and then function in pyroptosis has not been investigated. In this study, using LPS-treated BMECs as in vitro cell model, we first investigate the effects of UFL1 on NLRP3 inflammasome activation in LPS-induced BMECs. And then we further tested the hypothesis that UFL1 could alleviate pyroptosis through the inhibition of NLRP3 inflammasome activation.

2. Materials and methods

2.1. Animals

6 lactating Holstein cows with (n = 3) and without (n = 3) mastitis were used in this study. Pathological changes indicating clinical mastitis were signs of udder inflammation, including dolor, rubor, change of color, and visibly abnormal milk containing flakes. All experiments were carried out in accordance with the National Institutes of Health, and all experimental procedures were approved by the animal care committee of Nanjing Agricultural University.

2.2. Cell culture and treatments

Bovine mammary epithelial cells (BMECs, MAC-T) were a gift from Dr. Youping Sun (Harvard University). The BMECs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 200 U/mL of penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA)], and incubated at 37 ˚C in a humidified atmosphere containing 5% CO_2. The medium was changed every 48 h. Cells were primed with 200 ng/mL LPS (E. coli serotype O55:B5, Sigma-Aldrich) for 6 h and the treatments were performed in basal media without serum.

MAC-T were transfected with UFL1 siRNA, NLRP3 siRNA and negative control siRNA (GenePharma, Shanghai,China) using Lipofectamine 2000 (Invitrogen,Carlsbad, CA, USA) according to the manufacturer's instructions. The UFL1 siRNA sequence is 5′-GCAGCA GAA GCUUGUGAUATT-3′ and the antisense sequence is 5′-UAUCACA AGCUUCUGCUGCTT-3′; The NLRP3 siRNA sequence is 5′-GCGAGAAA UUCUACAGCUUTT-3′ and the antisense sequence is 5′ -AAGCUGUAG AAUUUCUCGCTT- 3′. After 48 h of transfection, the cells were then treated as indicated above. Successful depletion of UFL1 and NLRP3protein expression was confirmed by western blot analysis.

UFL1 plasmid was constructed in GenePharma (Shanghai,China) and was identified by JinsiruiBio Company (Nanjing, China). The amplified products were purified and cloned into pEX-3 vector. MAC-T cells were cultured at a confluency of 70–80% in 6-well dishes and transfected with 2 μg of the pcDNA3.1-UFL1 or the pcDNA3.1 empty vector in OptiMEM (Gibco, Carlsbad, CA, USA) using Lipofectamine 2000 (Invitrogen,Carlsbad, CA, USA) for 6 h following the specifications. After 48 h of transfection, the cells were then treated as indicated above. Successful overexpression of UFL1 protein expression was confirmed by western blot analysis.

2.3. Transmission electron microscopy (TEM)

At room temperature, BMECs were harvested and fixed in 2.5% glutaraldehyde. The fixed cells were postfixed in 1% osmium tetroxide, dehydrated using a graduated ethanol series (30, 50, 70, 80, 90, and 100%) for 10 min each, embedded in Epon (Energy Beam Sciences, Agawam, MA), sliced into ultrathin sections (50–60 nm) using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), and then stained with 3% uranylacetate and lead citrate.The ultrathin sectionswere observed under an H7500 transmission electron microscope (Hitachi,Tokyo, Japan).

2.4. LDH levels analysis

LDH levels in the supernatants were determined using a LDH cytotoxicity detection kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. The absorbance was read at 490 nm with a microplate reader.

2.5. Measurement of reactive oxygen species (ROS)

The level of ROS was measured using ROS assaykit (Nanjing Jiancheng, China) following the manufacturer's instructions and visualized under fluorescence microscope (Olympus, Tokyo, Japan). The fluorescence was measured using a microplate reader at excitation and emission wavelengths of 488 and 525 nm, respectively.

2.6. Determination of nitric oxide (NO) production

The NO concentration was determined using a Nitric Oxide Synthase Assay Kit (Beyotime) according to the manufacturer's protocols. The fluorescence intensity was read at 495 nm and 515 nm, respectively, using a Microplate Reader.

2.7. Measurement of Caspase1 activity

BMECs protein lysates were centrifuged at 18,000 rpm for 15 min at 4 ˚C and supernatants were quantified for Caspase1 activity using a commercial kit (Abcam, ab39412) according to the manufacturer's instructions.

2.8. Real-time quantitative PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the concentrations of RNA were measured by aNanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham,MA, USA). cDNA was synthesized using a Prime Script TM RT Master Mix (TaKaRa, Japan) according to the manufacturer's protocols. Real-time quantitative PCR was performed using standard protocols on an Applied Biosystem's 7500 H T Sequence detection system by SYBR ®Premix Ex Taq™ (TaKaRa, Japan). Gene expression data were normalized to the glyceraldehyde -3-phosphate dehydrogenase (GAPDH) by employing an optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method. The primer sense and antisense sequences were as follows: UFL1: forward 5′-TGTGGATCAG GTGGAAGCAT-3′, reverse 5′-TACAGCTGAAGCCTGTTTGC-3′; NLRP3: forward 5′-CTAGGCAACAACGACTTGGG-3′, reverse 5′-ACCGAGAAGG CTCA AAGACA-3′; iNOS: forward 5′-CTTGTTCTCGAGGTGCCCAT-3′, reverse 5′-GTCCCGGACTCCAACTTCTG-3′; GAPDH: forward 5′-CATGA CCACTTTGGCATCGT-3′, reverse 5′−CCATCCACAGTCTTCTGGGT-3′.

2.9. Immunoblot analysis

For Western Blot analysis, BMECs protein lysates were centrifuged at 15,000 rpm for 15 min at 4 ˚C and supernatants were quantified for total protein using a BCA protein assay kit (Beyotime, China) according to the manufacturer's instructions. Proteins were separated by SDS-PAGE and transferred to the PVDF membrane. The membranes were blocked in TBST containing 5% skim milk for 1 h and probed with the following primary antibodies: rabbit polyclonal antibody against UFL1 (1:1000, Proteintech), rabbit polyclonal antibody against pro-Caspase-1 (1:1000, Proteintech), rabbit polyclonal antibody against Caspase-1 (p10) (1:1000, Proteintech), rabbit polyclonal antibody against NLRP3 (1:1000, Novus), rabbit polyclonal antibody against pro-IL-1β (1:1000, Bioss), rabbit polyclonal antibody against IL-1β (1:1000, Bioss), rabbit polyclonal antibody against NF-κB P65(1:1000, Cell Signaling), rabbit

polyclonal antibody against Phospho-NF-κB P65 (1:1000, Cell Signaling) and rabbit polyclonal antibody against GAPDH (1:5000, Proteintech). The blots were incubated HRP-conjugated secondary antibodies and signals detected by enhanced chemiluminescence (ECL) Western blot detection reagents (Pierce, Rockford, IL, USA). Immunoblots were scanned and densitometry was performed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.10. Immunofluorescence staining

Cells were fixed, permeabilized, and blocked. And then incubated overnight at 4 °C with primary antibodies: rabbit polyclonal antibody against NLRP3 (1:200, Novus) and rabbit polyclonal antibody against Caspase-1 (1:200, Proteintech). After incubating overnight with primary antibodies, secondary fluorescent antibodies, Alexa 488- conjugated anti- rabbit Ab (1:400) were added for 1 h and DAPI was used for nuclear counter staining. The images were photographed using a fluorescence microscope (Olympus, Tokyo, Japan), and the fluorescence intensity was performed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.01 software (GraphPad Software Inc., SanDiego, CA). All data are shown as means ± SEM as indicated. The significance of the difference with different treatments was determined by an independent *t*-test. $P < 0.05$ was considered a significant difference.

3. Results

3.1. LPS increases NLRP3, Caspase-1 and IL-1β expression in vivo and in vitro

As mentioned above, NLRP3 inflammasome can be activated by pathogens and its activation will result in the cleavage of Caspase-1 into its p20 and p10 subunits and maturation of IL-1β, which mainly contribute to inflammatory damage(Schroder and Tschopp, 2010). Thus we explored the role of NLRP3 inflammasome activation in LPS-stimulated BMECs. As shown in Fig. 1A, the NLRP3 expression, activation of Caspase-1 and maturation of IL-1β were significantly increased in mammary gland of cows with clinical mastitis. Consistent with previous observations, the expression of NLRP3, Caspase-1 and IL-1β in were also increased in LPS-stimulated bovine mammary epithelial cells (Fig. 1B). These data reveal that NLRP3 inflammasome was activated after LPS treatment in BMECs, suggesting that NLRP3 inflammasome may play an important role in epithelial inflammation and dysfunction.

3.2. UFL1 modulates NLRP3 inflammasome-mediated IL-1β secretion and Caspase-1 activation in LPS-stimulated BMECs

According to the results of previous studies, UFL1 is a protein with many functions (Li et al., 2018; Zhang et al., 2015). As shown earlier and confirmed in our study, UFL1 can act as a therapeutic target in LPS-induced inflammatory response (Su et al., 2018). However, whether UFL1 could mediate LPS-induced NLRP3 activation remains unclear. We therefore explored the role of UFL1 in NLRP3 inflammasome-mediated Caspase-1 activation and IL-1β secretion in LPS-stimulated BMECs. As shown in Fig. 2, UFL1 silencing significantly induced the expression of NLRP3(Fig. 2B), western blot analysis showed an increase in NLRP3 expression in BMECs only treated with LPS (Fig. 2C and D), and this increase was significantly strengthened in UFL1-depleted cells following LPS challenge (Fig. 2C). Conversely, overexpression UFL1 remarkedly suppressed NLRP3 activation in LPS-stimulated BMECs compared to those of Con + LPS group (Fig. 2D). Consistent with NLRP3 expression, the activation of Caspase-1 and maturation of IL-1β were significantly increased in UFL1-depleted cells in response to LPS (Fig. 2C). By contrast, in LPS-stimulated UFL1-overexpressing cells, the expression of Caspase-1 and IL-1β was markedly attenuated than those of Con + LPS group (Fig. 2D). These results suggest that UFL1 could effectively attenuate Caspase-1 activation and IL-1β secretion through the inhibition of NLRP3 inflammasome activation.

3.3. UFL1 modulates ROS and nitric oxide production levels in LPS-stimulated BMECs

ROS production has been demonstrated to be one of the mechanisms involved in NLRP3 inflammasome activation and pyroptosis (Geng et al., 2015; Zhou et al., 2011). In addition, ROS production plays a crucial role in the production of nitric oxide, and overproduction of NO can damage cells and tissues, which is the major cause of inflammatory damage (Choi et al., 2015). We then detect the role of UFL1 in ROS and NO production. As shown in Fig. 3A, B and C, UFL1-silenced cells exhibited the higher levels of ROS, NO and iNOS production after LPS treatment than those of NC + LPS group. On the contrary, overexpression of UFL1 inhibited LPS-induced ROS and NO production

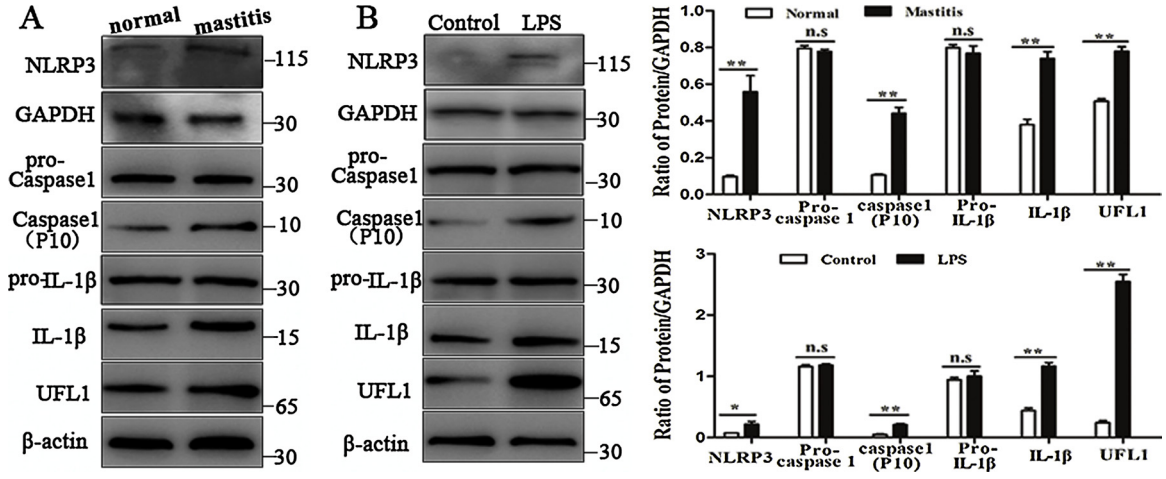

Fig. 1. LPS increases NLRP3, Caspase-1and IL-1β expression in *vivo* and *vitro*. (A) Representative immunoblots and quantification for analysis of NLRP3, pro-Caspase1, Caspase1, pro-IL-1β, mature IL-1β and UFL1 in mammary gland of bovine with clinical mastitis. (B) Representative immunoblots and quantification for analysis of NLRP3, pro-Caspase1, Caspase1, pro- IL-1β, mature IL-1β and UFL1 in LPS-stimulated BMECs. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

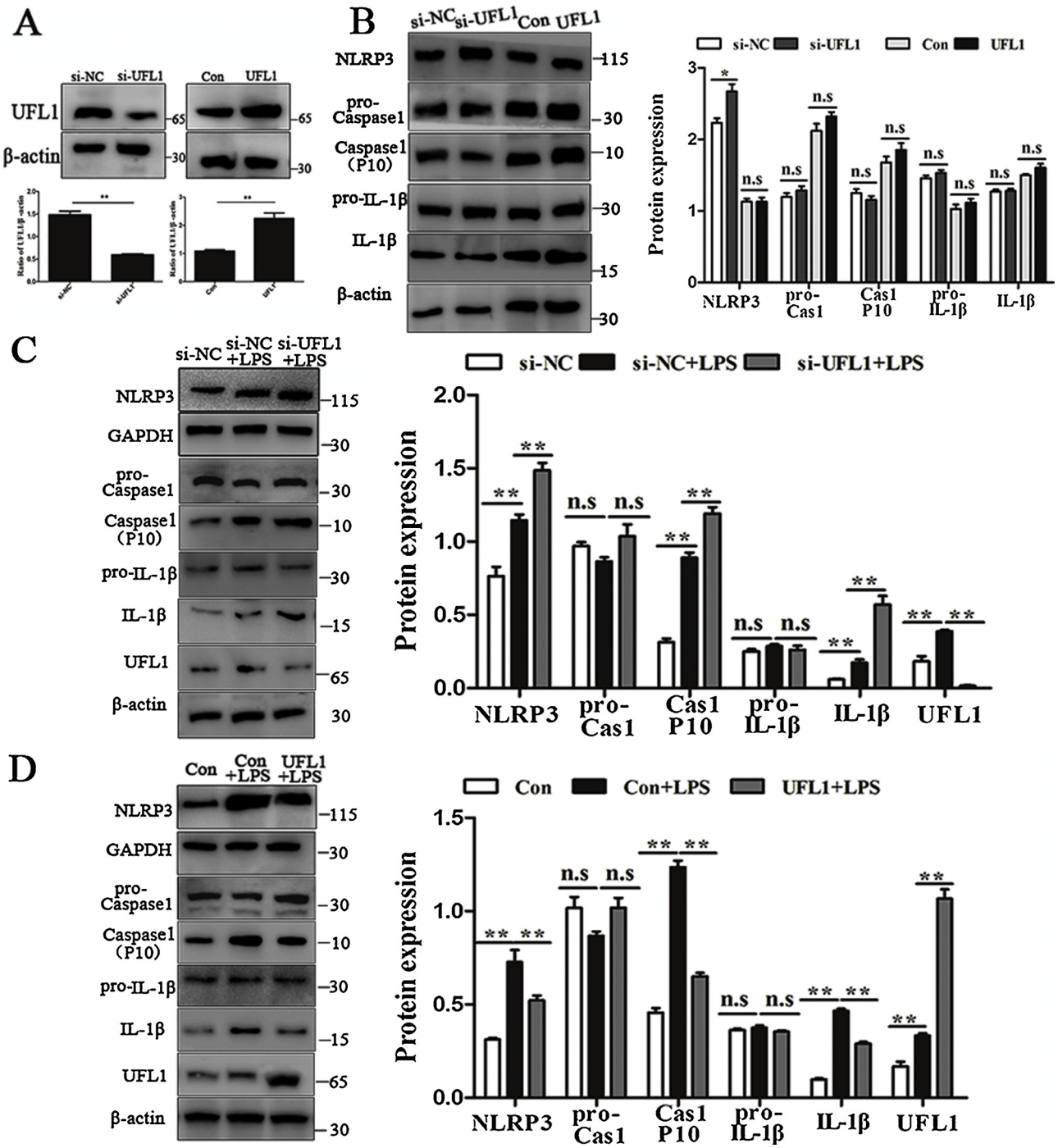

Fig. 2. UFL1 modulates NLRP3 inflammasome-mediated IL-1β secretion and Caspase-1 activation in LPS-stimulated BMECs. (A) Effect of UFL1 siRNA and overexpression plasmid on the expression of UFL1 in BMECs. (B) Effect of UFL1 on the protein expression of NLRP3, Caspase-1, IL-1β in BMECs. (C) Representative immunoblots and quantification for analysis of NLRP3, Caspase1 and IL-1β in UFL1-depleted cells stimulated with LPS. (D) Representative immunoblots and quantification for analysis of NLRP3, Caspase1 and IL-1β in LPS-stimulated BMECs transfected with UFL1 overexpression plasmid. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. * $p < 0.05$; ** $p <$ 0.01.

(Fig. 3D, E and F). These observations imply that UFL1 could inhibit the LPS-induced ROS, NO and iNOS production, the reduced ROS levels may likely suppresses NLRP3 inflammasome activation and contribute to the functional consequences of the inflammatory process.

3.4. UFL1 inhibits nuclear factor-kappaB (NF-κB) signaling

The essential for NLRP3 inflammasome activation is transcription factor NF-κB (Bauernfeind et al., 2009; Yu et al., 2017), numerous studies have shown that LPS through toll-like receptor 4 could activate the nuclear factor-kappaB (NF-κB), subsequently induce NLRP3 inflammasome and Caspase-1 activation, resulting in pyroptosis of many types of cell (Budai et al., 2013; He et al., 2016b; Qiao et al., 2012). We therefore investigated the effect of UFL1 on NF-κB signaling to better understand the mechanism underlying the activation of NLRP3 inflammasome. As shown in Fig. 4A, UFL1 depletion significantly increased the expression of Phospho-NF-κB P65, whereas overexpression of UFL1 led to a marked reduction in the expression of Phospho-NF-κB

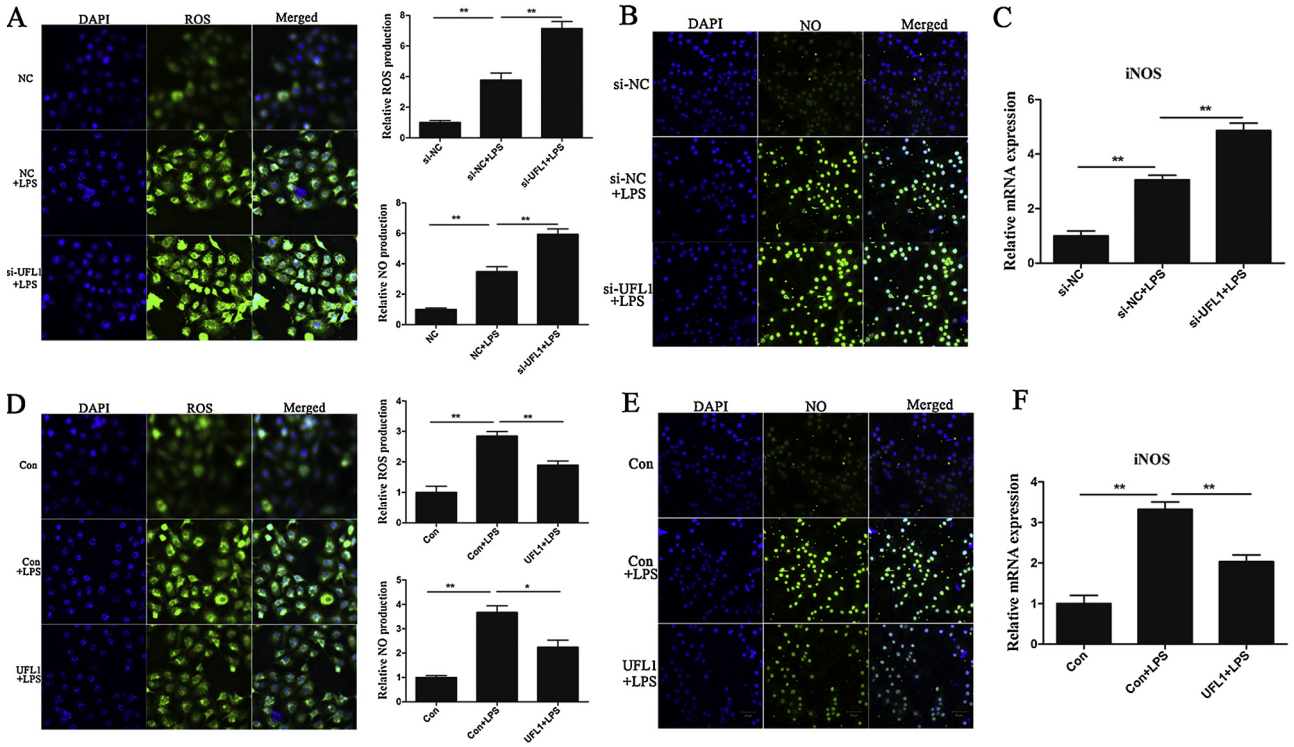

Fig. 3. UFL1 modulates ROS and NO production levels in LPS-stimulated BMECs. (A), (B), (C) Effect of UFL1 siRNA on the level of ROS, NO production and iNOS mRNA expression in LPS challenged BMECs. (D), (E), (F) Effect of UFL1 overexpression on the level of ROS, NO production and iNOS mRNA expression in LPS challenged BMECs. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

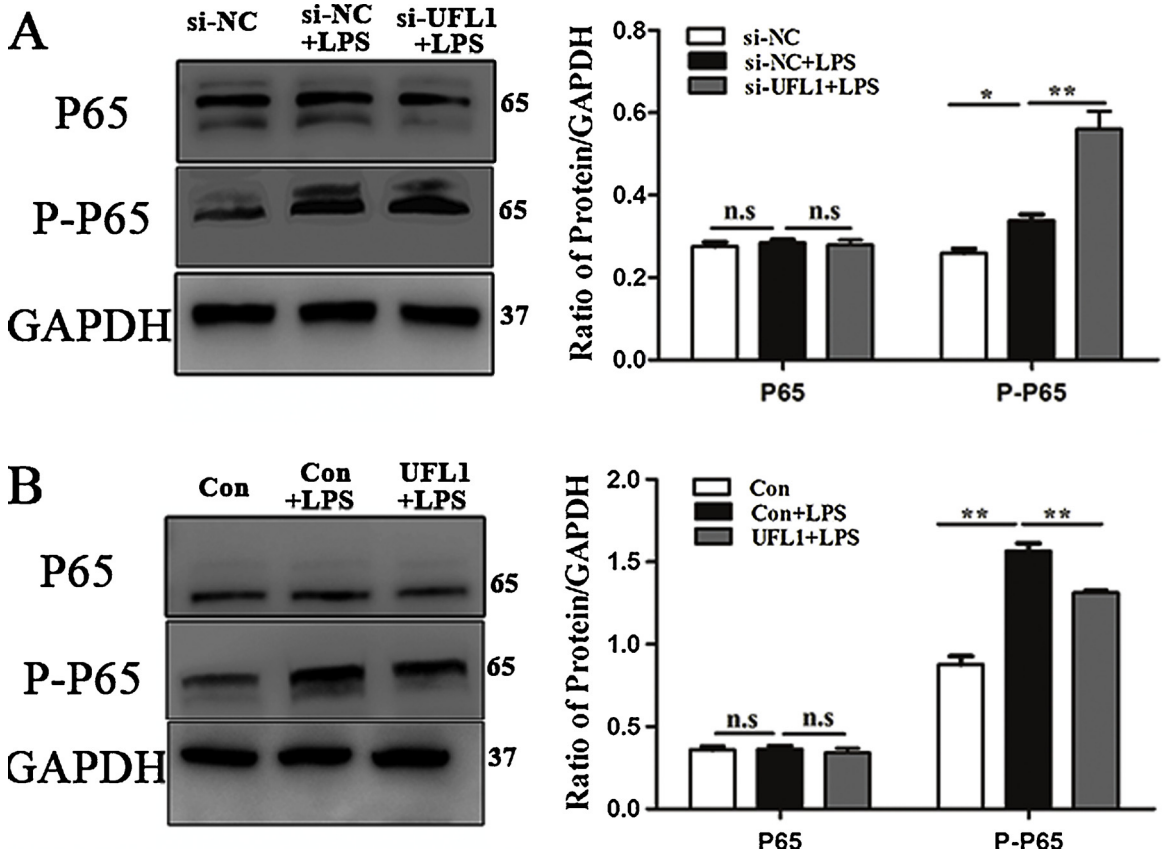

Fig. 4. UFL1 inhibits nuclear factor-kappaB (NF-κB) signaling. (A) Representative immunoblots and quantification for analysis of NF-κB P65 and Phospho-NF-κB P65 in UFL1 siRNA-transfected BMECs stimulated with LPS. (B) Representative immunoblots and quantification for analysis of NF-κB P65 and Phospho-NF-κB P65 in BMECs transfected with UFL1 overexpression plasmid. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

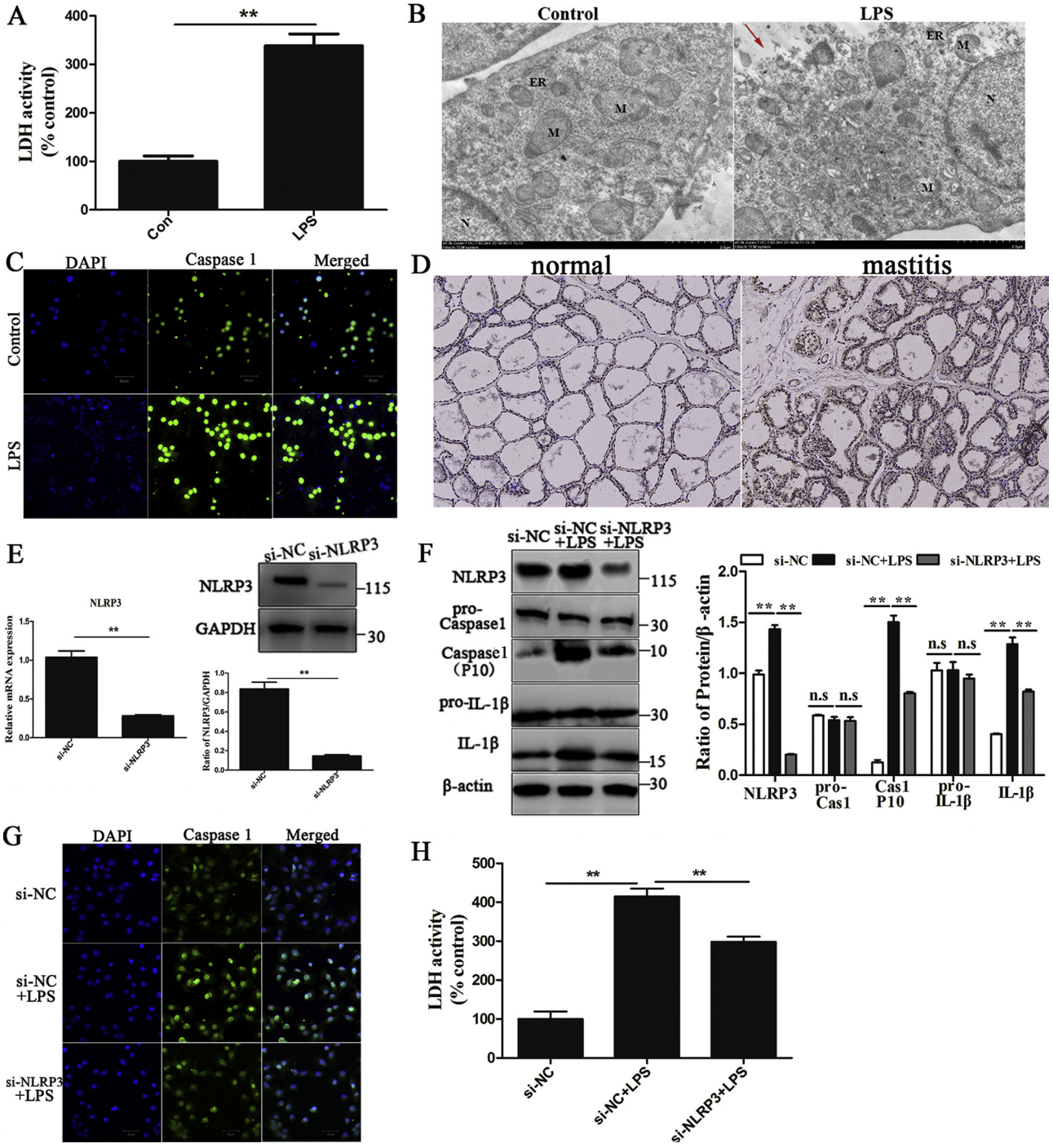

Fig. 5. NLRP3 inflammasome activation mediates pyroptosis in LPS-stimulated BMECs. (A) LDH release after treatment with LPS in mammary epithelial cells. (B) TEM images of LPS-treated BMECs. Red arrowhead: plasma membrane, M: mitochondria, N: nucleus, ER: endoplasmic reticulum. (C) Immunofluorescent staining of BMECs with anti-Caspase1 antibody in LPS challenged BMECs. (D) Caspase-1 levels using immunohistochemical staining in normal cows and cows with mastitis. (E) Efficiency of siRNA against NLRP3 in BMECs. (F) Representative immunoblots and quantification for analysis of Caspase1 and IL-1β in NLRP3-depleted cells stimulated with LPS. (G) Immunofluorescent staining of BMECs with anti-Caspase1 antibody in NLRP3-depleted cells stimulated with LPS. (H) LDH release in NLRP3-depleted cells stimulated with LPS. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. ** $p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

P65 (Fig. 4B). These results suggest that UFL1 could inhibit NLRP3 inflammasome activation via downregulation of NF-κB signaling.

3.5. NLRP3 inflammasome activation mediates pyroptosis in LPS-stimulated BMECs

As previously mentioned, NLRP3 inflammasome activation causes a rapid, pro-inflammatory programmed cell death called pyroptosis (He et al., 2016a). We then further verified the effect of NLRP3 Inflammasome Activation on LPS-induced pyroptosis in Bovine Mammary Epithelial Cells. In our present study, we firstly determined whether LPS treatment caused pyroptosis in BMECs, cell pyroptosis was detected by Caspase-1 activation and LDH release. As shown in Fig. 5, exposure to LPS resulted in an increase in LDH release in mammary epithelial cells

(Fig. 5A). Notably, cell pyroptosis was also discovered and confirmed under conventional TEM, as indicated by the plasma membrane rupture and cytoplasmic swelling (Fig. 5B). Moreover, immunohistochemical and immunofluorescence staining were performed to examine Caspase-1 activation. Results showed that Caspase-1 activation was considerably promoted in mammary tissue of cows with mastitis and LPS-stimulated BMECs (Fig. 5C and D). In view of these results that LPS treatment caused cell death, we then knocked down the expression of NLRP3 by siRNA to assess the role of NLRP3 inflammasome in LPS-induced pyroptosis in mammary epithelial cells. As shown, siRNA against NLRP3 successfully down-regulated the protein levels of NLRP3 in both normal and LPS-treated BMECs (Fig. 5E and F). Also, the siRNA of NLRP3 diminished Caspase-1 expression and IL-1β production in LPS-stimulated BMECs, indicating that LPS-triggered NLRP3 inflammasome activation was suppressed (Fig. 5F). In addition, silencing of NLRP3 abrogated cell pyroptosis induced by LPS treatment as evidenced by decreased Caspase-1 and reduced LDH release (Fig. 5G and H). These results indicated that targeting NLRP3 inflammasome activation may serve as effective strategies to diminish cell damage in inflammatory response.

3.6. UFL1 modulates NLRP3 inflammasome activation-mediated Pyroptosis in LPS-stimulated BMECs

As confirmed in our present study, UFL1 could modulate LPS-induced NLRP3 inflammasome activation. We therefore investigated the role of UFL1 in NLRP3 Inflammasome Activation-mediated Pyroptosis. We first performed CCK8 assays to evaluate cell viability after UFL1 silencing or UFL1 overexpression in LPS-stimulated BMECs. As shown in Fig. 6A, the viability analysis revealed that knockdown of UFL1 resulted in a significant further decrease in the viability of LPS-challenged BMECs. However, UFL1 overexpression resulted in a much higher level of cell viability compared with that in the Con + LPS group (Fig. 6E). Strikingly, silencing of UFL1 also exhibited a much higher level of LDH release and Caspase 1 activation than those of NC + LPS group (Fig. 6B, C and D). On the contrary, overexpression of UFL1 showed significantly reduced LDH release and Caspase 1 activation compared with those of Con + LPS group (Fig. 6F, G and H). These findings revealed an important role of UFL1 in NLRP3 inflammasome activation-mediated pyroptosis in LPS-stimulated bovine mammary epithelial cells, which suggested that UFL1 could act as an effective strategy to diminish cell damage in inflammatory response.

4. Discussion

In the present study, we unraveled novel roles of UFL1 in NLRP3 inflammasome activation and pyroptosis regulation in LPS-stimulated bovine mammary epithelial cells. The experimental results here demonstrated that UFL1 depletion aggravated the activation of NLRP3 and expression of Caspase-1 and IL-1β. Overexpression of UFL1 suppressed the activation of NLRP3 and expression of Caspase-1 and IL-1β. The suppression of NLRP3 inflammasome activation by UFL1 was partly mediated through the regulation of NF-κB signaling and ROS production. Furthermore, UFL1 overexpression could alleviate NLRP3 inflammasome activation-mediated pyroptosis in LPS-stimulated BMECs. These findings indicate that UFL1 can moudulate NLRP3 inflammasome activation and serve as effective strategies to diminish cell damage in inflammatory response by targeting NLRP3 inflammasome activation.

The release of LPS by Gram-negative bacteria is a major environmental pathogen causing bovine mastitis, which leads to mammary tissue and cell damage (Opal, 2007). BMECs in mammary glands are the first line of protection against contact with invading pathogens. Several previous studies have found that the excessive inflammation response stimulated by LPS would injure BMECs, even result in the death of the cell(Liu et al., 2014; Wellnitz et al., 2016; Wu et al., 2016). The inflammatory response of BMECs is initiated following the detection of pathogens via specialized pattern recognition receptors (PRRs), e.g., toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs)(Porcherie et al., 2012; Zhu et al., 2012). Activation of TLRs or NLRs leads to NLRP3 inflammasome activation, resulting in upregulation in the transcription of Caspase-1 and proinflammatory cytokine genes (Latz et al., 2013b; Rathinam et al., 2012), therefore trigger adaptive immune response and induce pyroptosis. It has been observed that treatment of various cell types with LPS triggered the activation of NLRP3 inflammasome (Hu et al., 2017; Li et al., 2016; Luo et al., 2017; Wu et al., 2016). Consistent with these in *vitro* results, we

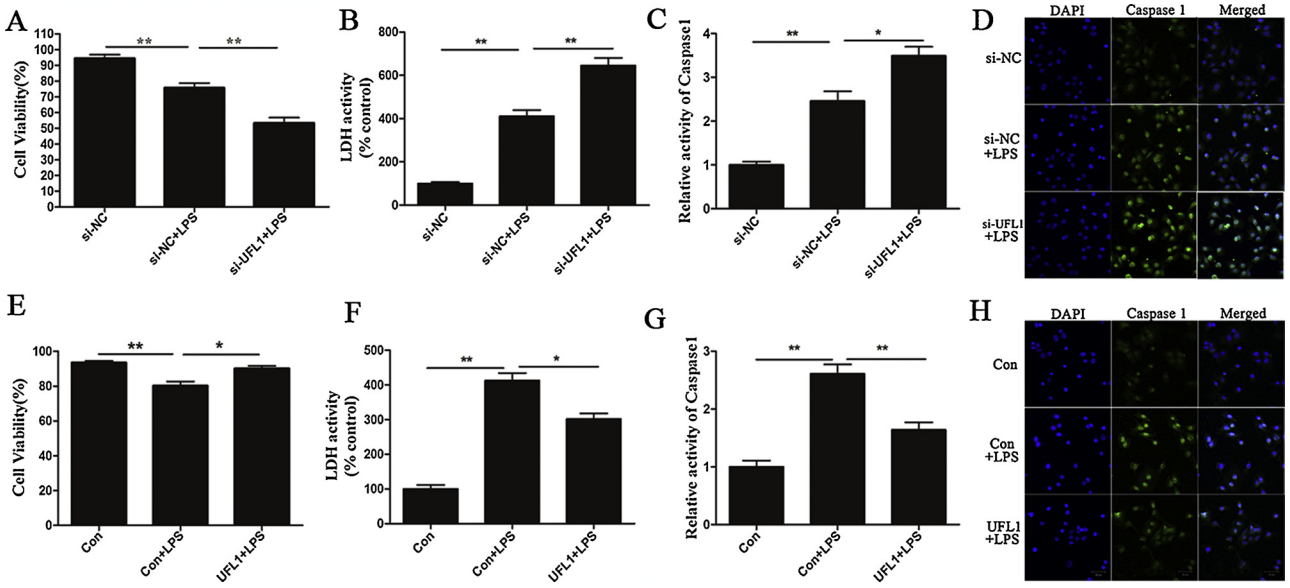

Fig. 6. UFL1 modulates NLRP3 inflammasome activation-mediated pyroptosis in LPS-stimulated BMECs. (A), (B), (C) Cell viability, LDH release and Caspase1 activity in UFL1-depleted cells stimulated with LPS. (D) Immunofluorescent staining of BMECs with anti-Caspase1 antibody in UFL1-depleted cells stimulated with LPS. (E), (F), (G) Cell viability, LDH release and Caspase1 activity in LPS-stimulated BMECs transfected with UFL1 overexpression plasmid. (H) Immunofluorescent staining of BMECs with anti-Caspase1 antibody in BMECs transfected with UFL1 overexpression plasmid. Data are presented as the means ± the standard errors of the mean (SEM) of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

found that the expression of NLRP3, Caspase-1 and IL-1β protein were increased in mammary tissue of cows with mastitis and LPS-stimulated BMECs (Fig. 1A and B). These results suggest that LPS treatment induce the activation of the NLRP3 inflammasome and subsequently the maturation of Caspase-1 as well as IL-1β production in BMECs.

Activation of the NLRP3 inflammasome is thought be regulated at both the transcriptional and post-translational levels (Jo et al., 2016). The essential for NLRP3-inflammasome activation is transcription factor NF-κB, which is required for production of IL-1β and important for inflammasome priming and assembling(Schroder and Tschopp, 2010). As stated above, UFL1 was proved to regulate celluar life activities by negatively regulating NFκB, a master controller of inflammation. Strikingly, our results confirmed that the suppression of NLRP3 inflammasome activation by UFL1 involves the downregulation of NFκB signaling (Fig. 4). Besides, inflammation involves many complex interactions between cellular and inflammatory mediators. During the inflammatory process, significant amount of ROS and NO are produced, the excessive production of ROS and NO induces cell injury and cell death. Furthermore, ROS was confirmed to be another important factor which could mediate LPS-activated NLRP3 inflammasome activation (Harijith et al., 2014; He et al., 2016b), the activation of NLRP3 inflammasome can be induced by ROS production. UFL1, the most recently identified protein, can act as a key regulator of cellular stress response. Loss of UFL1 can lead to accumulation of ROS and mitochondrial mass, enhance cell death (Zhang et al., 2015). Here we found that the UFL1-silenced cells exhibited a much higher levels of ROS and NO (Fig. 3A, B and C), overexpression of UFL1 inhibited LPS-induced ROS and NO production (Fig. 3D, E and F), the results of the experiment of ROS production is consistant with previous studes(Wu et al., 2010; Zhang et al., 2015)· These findings revealed that UFL1 could modulate NLRP3 inflammasome activation in LPS-stimulated bovine mammary epithelial cells. Whether the suppression of NLRP3 inflammasome activation by UFL1 involves additional signal pathway still needs further research.

To date, the mechanism of LPS-induced epithelial injury has mainly concentrated on proinflammatory effects, oxidative stress and apoptosis, while the process of pyroptosis is rarely mentioned. As many studies on LPS-induced inflammatory responses have focused on NLRP3 inflammasome activation and pyroptosis occurs after activation of Caspase-1, we first verified the role of the NLRP3 inflammasome in the pyroptosis induced by LPS in BMECs. Results showed that LPS treatment induced an increase in LDH release in BMECs (Fig. 5A). Elevated LDH indicated that, in BMECs, cell death may be a component of the cytotoxicity induced by LPS. In addition, silencing NLRP3 diminished Caspase-1 expression and IL-1β production (Fig. 5F), this finding was consistent with previous studies, in which the authors found that both Caspase-1 and IL-1β production were decreased after knocking down of NLRP3 (Chen et al., 2016; Liu et al., 2017; Lu et al., 2016). More vitally, NLRP3 depletion significantly decreased the proportion of pyroptosis in LPS-stimulated BMECs (Fig. 5G and H). These results indicated that targeting NLRP3 inflammasome activation may serve as effective strategies to diminish cell damage in inflammatory response. According to the above results that UFL1 could modulate NLRP3 inflammasome activation (Fig. 2), we then explored the effect of UFL1 on NLRP3 inflammasome activation-mediated pyroptosis. As expected, UFL1 could protect against LPS-induced pyroptosis via modulation of NLRP3 inflammasome activation (Fig. 6). In recent years, a tremendous amount of efforts has been devoted to understanding the mechanisms of pyroptosis in many diseases and to determine the genes and pathways involved in this process. However, the molecular components regulating pyroptosis in bovine mastitis remain largely unknown. In the present study, we confirmed that pyroptosis was induced in mammary tissue of cows with mastitis and LPS-stimulated BMECs, and UFL1 could alleviate NLRP3 inflammasome activation-mediated pyroptosis. However, the molecular mechanisms involved in these processes should be further elucidated.

Taken together, our results provide the first evidence that UFL1 could modulate NLRP3 inflammasome activation and protects against pyroptosis in LPS-stimulated BMECs. The findings from the present study indicate the essential role of UFL1in bovine mastitis and will greatly improve our understanding of the role of NLRP3 inflammasome in bovine mastitis and may provide an effective therapeutic approach for the conditions associated with NLRP3 inflammasome activation.

Conflicts of interest

The authors declare that they have no conflict of interests.

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