



NLRP3 inflammasome plays an important role in caspase-1 activation and IL-1 β secretion in macrophages infected with *Pasteurella multocida*

Rendong Fang^{a,1}, Huihui Du^{a,b,1}, Guihua Lei^{a,1}, Yajing Liu^a, Siwei Feng^a, Chao Ye^a, Nengzhang Li^a, Yuanyi Peng^{a,*}

^a College of Animal Science and Technology, Southwest University, Chongqing, 400715, China

^b College of Biology and Food Engineering, Chongqing Three Gorges University, Chongqing, 404100, China

ARTICLE INFO

Keywords:

Pasteurella multocida
NLRP3 inflammasome
IL-1 β
Syk

ABSTRACT

Pasteurella multocida is a Gram-negative bacterium that is responsible for a variety of diseases in birds and mammals, including humans. We have previously reported that the *P. multocida* serotype A strain PmCQ2 causes severe lung pneumonia in bovines. Transcriptomic analysis showed that many genes related to the immune response were significantly upregulated in the lungs of mice infected with *P. multocida* compared with uninfected mice. However, the mechanism by which *P. multocida* induces host inflammatory cytokine secretion is poorly understood. In this study, the mechanism of caspase-1 activation and subsequent IL-1 β secretion in macrophages infected with *P. multocida* was elucidated. The nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome was shown to be involved in inducing this cellular response. Compared with wild-type macrophages, *Nlrp3*^{-/-} macrophages exhibited a clear decrease in caspase-1 activation and IL-1 β secretion in response to *P. multocida* infection. Furthermore, spleen tyrosine kinase (Syk) was indicated to be involved in IL-1 β secretion, possibly by regulating the NLRP3 inflammasome. Our results provide new insight into the host proinflammatory immune response against *P. multocida* and the critical involvement of the NLRP3 inflammasome in this activity.

1. Introduction

Pasteurella multocida (*P. multocida*) is a gram-negative bacterial pathogen that causes a variety of diseases in birds and mammals, including humans. *P. multocida* is classified into five capsular serotypes (A–F) and 16 Heddleston serotypes based on the lipopolysaccharide (LPS) antigens (Wilkie et al., 2012). *P. multocida* serotype A isolates are common residents of the upper respiratory tract and are associated with pneumonia and bovine respiratory disease complex (BRDC) (Dabo et al., 2007). Although this bacterium has been known for decades, little is known about the pathogenesis of pasteurellosis, and the host immune responses against *P. multocida* infection are also poorly understood. The inadequate understanding of *P. multocida*-host interactions has limited the development of an effective vaccine and new therapeutic strategies against *P. multocida*-driven diseases.

Previous reports have shown that a mouse model can potentially be used for bovine pasteurellosis, and an intranasal challenge route may be used to mimic the natural route of infection (Hodgson et al., 2013; Kharb and Charan, 2013; Pors et al., 2016). It has been reported that

mouse *P. multocida* infections can result in strong secretion of proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin 1 β (IL-1 β), and interleukin 6 (IL-6) (Praveena et al., 2010). Recently, we also reported the responses to *P. multocida* infection in murine lungs by transcriptomic analysis (Wu et al., 2017). Our results indicated that *P. multocida* can induce inflammation in the lungs, and genes related to immune responses, including genes encoding pattern recognition receptor (PRR), inflammatory cytokines and chemokines, were significantly upregulated. These inflammatory responses might play important roles in *P. multocida* infections. However, the mechanism of inflammatory cytokine production during *P. multocida* infection is still unclear.

The innate immune system provides a first line of defense to protect the host against infections by pathogens. Macrophages sense conserved microbial structures and induce a proinflammatory response via the secretion of cytokines. IL-1 β is a pleiotropic cytokine that is a master mediator of inflammation and that plays an important role in host defense against bacterial infection and tissue injury (Borthwick, 2016; Manoranjan Sahoo et al., 2011). Due to the importance of IL-1 β , the

* Corresponding author at: College of Animal Science and Technology, Southwest University, Chongqing, 400716, China.

E-mail address: pyy2002@sina.com (Y. Peng).

¹ These authors contributed equally to this work.

production and activity of this cytokine have been extensively studied. Inflammasome assembly and caspase-1 activation have been reported to be the predominant processes responsible for IL-1 β maturation and secretion, especially in macrophages. The nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is the most extensively studied inflammasome; NLRP3 responds to diverse stimuli and forms an inflammasome complex with pro-caspase-1 and apoptosis-associated speck-like protein containing CARD (ASC) (Jo et al., 2016; Wen et al., 2013). The activation of caspase-1 enables the cleavage of the proinflammatory cytokines IL-1 β and IL-18 and the subsequent secretion of mature IL-1 β and IL-18 (Sansonetti et al., 2000).

In this study, we focused on elucidating the mechanism of IL-1 β secretion in macrophages infected by *P. multocida* and demonstrated the involvement of the NLRP3 inflammasome and caspase-1 activation in the maturation and secretion of IL-1 β , which provides new insight into the host proinflammatory immune response against *P. multocida*.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from the Chongqing Academy of Chinese Materia Medica (Chongqing, China). *Casp1*^{-/-}, *Asc*^{-/-}, *Nlrp3*^{-/-}, *Nlr4*^{-/-}, *Aim2*^{-/-} and *Tlr4*^{-/-} mice were kindly provided by Dr. Feng Shao from NIBS (National Institute of Biological Sciences, Beijing). The mice were maintained in specific pathogen-free conditions with automated water delivery and free food intake and were used at 8–10 weeks of age. All of the animal experiments were approved by the Animal Ethics and Research Committee of Southwest University (Permit No. 11-1025), Chongqing, China.

2.2. Bacterial strains

The highly virulent bovine *P. multocida* capsular type A isolate PmCQ2 (GenBank accession number: LIUN00000000) was isolated from the lungs of calves with pneumonia in Chongqing, China (Du et al., 2016). The bacteria from the stock stored at -80 °C were incubated and activated on Martin's agar plates at 37 °C for 18–24 h. Then, the bacterial colony was incubated with shaking in 5 ml RPMI 1640 medium (Gibco, USA) supplemented with 10% FCS (Gibco, USA) at 37 °C for 8–10 h.

2.3. Preparation of peritoneal macrophages

Mice were injected intraperitoneally (i.p.) with 4% thioglycolate broth (Eiken, Japan), and peritoneal exudate cells (PECs) were collected 3 days later. The PECs were suspended in RPMI 1640 medium containing 10% FCS. The cells were counted with a hemocytometer and incubated in 48-well microplates at a density of 2×10^5 cells/well at 37 °C plus 5% CO₂. The nonadherent cells were removed after 2 h.

2.3.1. ELISA

Adherent macrophages were washed with PBS for three times, the medium was recovered with RPMI 1640 medium containing 10% FCS and infected with *P. multocida* at a multiplicity of infection (MOI) of 1 for 9 h, and then 100 μ g/ml ciprofloxacin (Solarbio, Beijing, China) was added to the cultures for an additional 15 h incubation. The culture supernatants were collected and used. Inhibitors of JNK (SP600125), Syk (R406), p38MAPK (SB203580), NF- κ B (BAY11-7082), caspase (Z-VAD-FMK) were purchased from Beyotime (Beijing, China), P2 $\times$ 7 receptor inhibitor (A438079, Selleck Chemicals). All inhibitors were added to cultures 1 h before infection. Levels of secreted cytokines in the culture supernatants were determined by a two-site sandwich enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-1 β , TNF- α , IL-12p40, IL-6, IL-1 α were purchased from eBioscience (San Diego, CA),

and ELISA kit for IL-18 was purchased from Thermo Scientific.

2.4. Western blot analysis

PECs were cultured in 12-well plates at a density of 1×10^6 cells/well in RPMI 1640 containing 10% FCS at 37 °C for 2 h. After washing with PBS three times, the culture medium was replaced with Opti-MEM (Gibco, USA), and the adherent cells were infected with *P. multocida* at an MOI of 1. Supernatants were collected after 24 h of infection, and the cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (Beyotime, China). Inhibitors of JNK (SP600125) and Syk (R406) were added to cultures 1 h before infection, respectively. The culture supernatants were concentrated using 20% (w/v) trichloroacetate. The precipitates and cell lysates were subjected to SDS-PAGE and subsequently transferred to 0.2 μ m polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were immunoblotted with an anti-caspase-1 antibody (AdipoGen, USA), anti-IL-1 β antibody (Proteintech, China) and anti- β -actin antibody (Proteintech, China).

2.5. Quantitative RT-PCR analysis

Total cellular RNA was extracted from the adherent PECs by using an RNApure pure Cell/Bacteria Kit (TIANGEN, Beijing, China). cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad, California, USA), and quantitative real-time RT-PCR was performed using a CFX96 instrument (Bio-Rad, California, USA). Primers for RT-PCR as follows: *Nlrp3* Fw: 5'-GAG TTC TTC GCT GCT ATG T-3'; Rv: 5'-ACC TTC ACG TCT CGG TTC -3'; *IL-1 β* Fw: 5'-GAA ATG CCA CCT TTT GAC AGT G -3'; Rv: 5'-TGG ATG CTC TCA TCA GGA CAG -3'; β -actin: Fw: 5'-GTC CAC CTT CCA GCA GAT GT -3'; Rv: 5'-GAA AGG GTG TAA AAC GCA GC -3'.

2.6. Detection of macrophage-associated and phagocytosed *P. Multocida*

Adherent macrophages were infected with *P. multocida* at an MOI of 1, washed with chilled PBS three times to remove non-associated bacteria and lysed in PBS containing 0.1% Triton X-100 every 3 h until 24 h. The cell lysates were diluted with PBS and grown on Martin's agar plates at 37 °C for 18–24 h. To count the phagocytosed bacteria, the macrophages were infected with *P. multocida* as described above and cultured for an additional 30 min in the presence of 100 μ g/ml ciprofloxacin. Then, the macrophages were washed with PBS, and the number of bacteria in the cell lysates was determined. To inhibit the phagocytosis of *P. multocida* by macrophages, cytochalasin B was added to the cell cultures at a final concentration of 10 μ g/ml, P2X7 receptor inhibitor (A438079, 100 μ M) and an equal volume of DMSO was added as control groups.

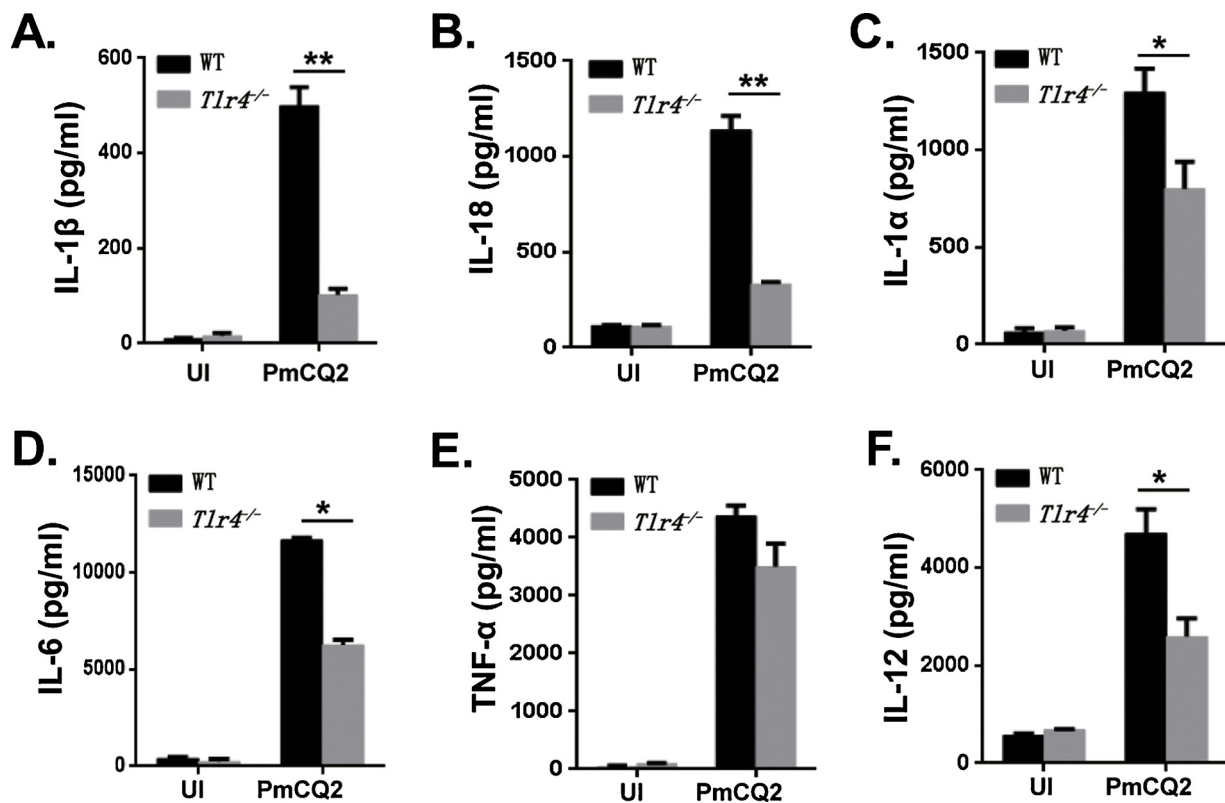
2.7. Statistical analysis

For comparisons between two groups, Student's *t*-test was performed with SPSS Statistics 22.0 software. Statistical significance was determined as **p* < 0.05 and ***p* < 0.01.

3. Results

3.1. Cytokine secretion by macrophages infected with *P. Multocida* in vitro

To evaluate the proinflammatory response induced by *P. multocida* in vitro, peritoneal macrophages from C57BL/6 WT and *Tlr4*^{-/-} mice were infected with *P. multocida*, and the levels of cytokines in the culture supernatants were assayed. Compared with the culture supernatant of the uninfected group, the culture supernatant of the PmCQ2-infected group showed significant increases in the levels of IL-1 β , IL-18, IL-1 α , IL-6, TNF- α and IL-12p40 by ELISA. Macrophages from TLR4 knockout mice resulted in largely decrease in secretion of these cytokines upon *P. multocida* infection, especially for IL-1 β and IL-18 (Fig. 1A–F). These



PECs from C57BL/6 WT and *Tlr4*^{-/-} mice were left uninfected (UI) or infected with *P. multocida* at an MOI of 1 for 9 h, and then ciprofloxacin (final concentration of 100 µg/ml) was added to the cultures for an additional 15 h incubation. The supernatants were collected at 24 h postinfection. The levels of IL-1β (A), IL-18 (B), IL-1α (C), IL-6 (D), TNF-α (E) and IL-12p40 (F) in the culture supernatants were determined by ELISA. The results are representative of three independent experiments. Statistical significance was determined by Student's *t*-test (**p* < 0.05, ***p* < 0.01).

results demonstrated that the proinflammatory response of the macrophages was activated by *P. multocida* infection, and the induction of these proinflammatory cytokines majorly mediated by the TLR4 receptor, but other receptors might be also involved.

3.2. NLRP3 inflammasomes play a critical role in caspase-1 activation and IL-1β secretion

It is well known that inflammasomes play an important role in the activation of caspase-1 and the subsequent secretion of IL-1β. To test whether inflammasomes are involved in the proinflammatory responses induced by *P. multocida*, macrophages from C57BL/6 WT, *Casp1*^{-/-}, *Asc*^{-/-}, *Nlrp3*^{-/-}, *Nlr4*^{-/-} and *Aim2*^{-/-} mice were infected with *P. multocida*. Caspase-1 activation was completely abolished in *Casp1*^{-/-}, *Asc*^{-/-} and *Nlrp3*^{-/-} macrophages, and correspondingly, IL-1β secretion was almost undetectable in these knockout macrophages. A deficiency in AIM2 or NLR4 did not affect caspase-1 activation or IL-1β secretion (Fig. 2A–B). The secreted levels of IL-6 and TNF-α, which are not dependent on an inflammasome and caspase-1 were detected as control, the results showed that no differences among all the groups for these two cytokines (Fig. 2C–D). Taken together, these results suggested that the NLRP3 inflammasome is essential for caspase-1 activation and IL-1β maturation and secretion in *P. multocida*-infected macrophages.

3.3. Expression of pro-IL-1β in macrophages induced by *P. Multocida* is partially dependent on TLR4 but independent of the NLRP3 inflammasome

NLRP3 inflammasome activation requires two steps. The NF-κB-mediated expression of NLRP3 is upregulated in the priming step, and then inflammasome assembly is triggered by a second signal (Bauernfeind et al., 2009). Functional IL-1β secretion by macrophages

also requires two steps. First, pro-IL-1β is synthesized after a stimulus, and second, pro-IL-1β is cleaved by activated caspase-1 into its mature form and secreted. We examined whether TLR4 and the NLRP3 inflammasome participates in the expression of pro-IL-1β in macrophages infected with *P. multocida*. The results showed that pro-IL-1β expression was significantly increased after *P. multocida* infection, TLR4 knockout macrophages showed partially decrease of the expression level, but not totally abrogated as that of LPS stimulated group (Fig. 3A). The expression levels of both NLRP3 was also significantly increased after *P. multocida* infection (Fig. 3B). However, comparable levels of pro-IL-1β were detected in macrophages from WT, *Casp1*^{-/-}, *Asc*^{-/-} and *Nlrp3*^{-/-} mice (Fig. 3C). These results suggested that TLR4 is partially involved, but the NLRP3 inflammasome is not involved in the expression of pro-IL-1β in *P. multocida*-infected macrophages.

3.4. Phagocytosis of *P. multocida* by macrophages is important for IL-1β secretion

The NLRP3 inflammasome functions in the cytosolic space, so how could *P. multocida* infection activate the NLRP3 inflammasome. We hypothesized that *P. multocida* was recognized by NLRP3 after being phagocytosed by macrophages. To test whether phagocytosis is a prerequisite for caspase-1-dependent IL-1β secretion, we first determined the fate of *P. multocida* in macrophages postinfection. The numbers of macrophage-associated and macrophage-phagocytosed *P. multocida* were counted at different time points after infection, and the results showed that the number of macrophage-associated *P. multocida* peaked at 6 h postinfection (Fig. 4A). The number of *P. multocida* phagocytosed by macrophages increased and then declined; at 9.5 h after infection, the quantity of phagocytosed *P. multocida* peaked (Fig. 4B). To elucidate whether phagocytosis is required for the secretion of IL-1β by

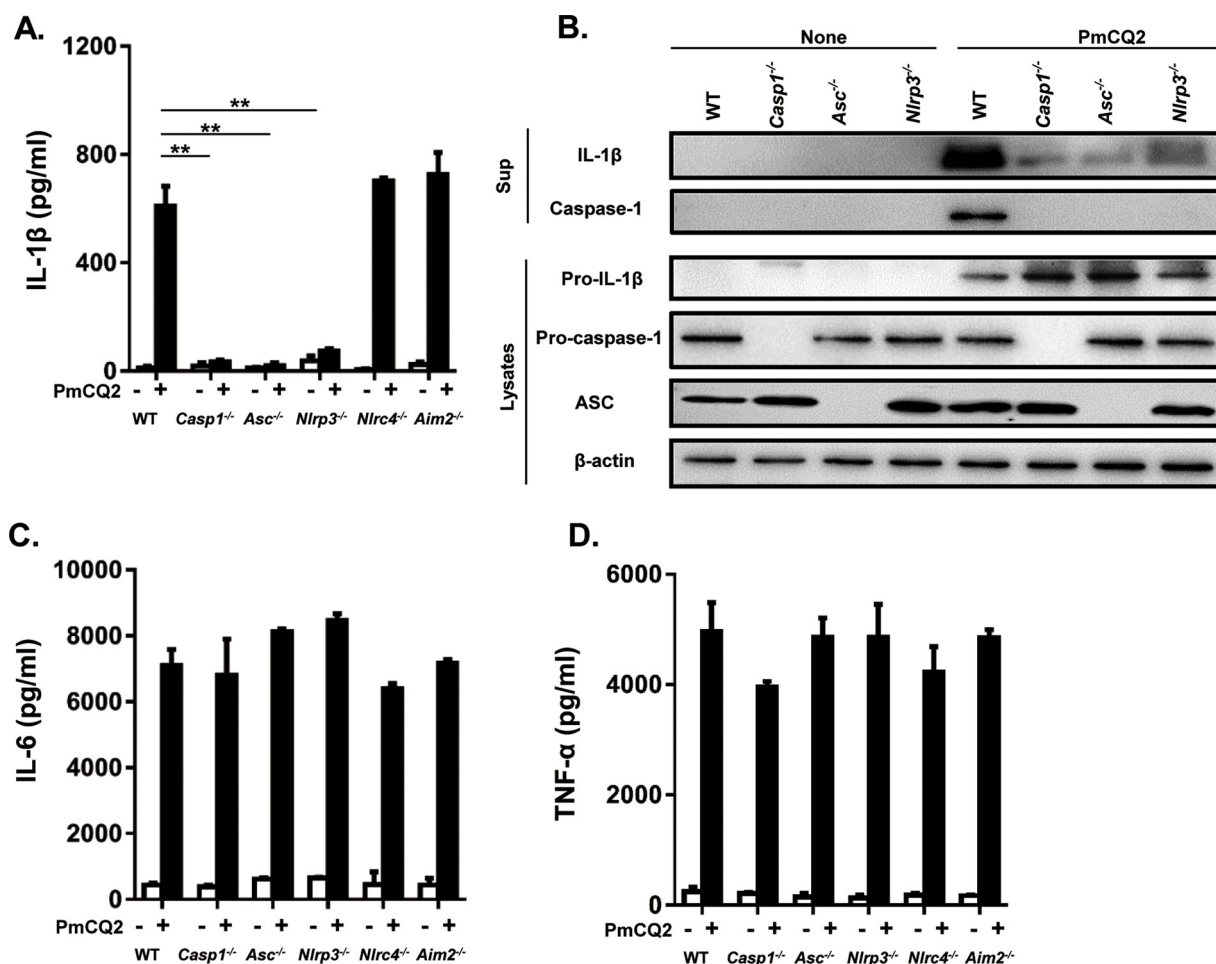


Fig. 2. NLRP3 inflammasome is essential for caspase-1 activation and IL-1β secretion in *P. multocida*-infected macrophages.

PECs from C57BL/6 WT, *Casp1*^{-/-}, *Asc*^{-/-}, *Nlrp3*^{-/-}, *Nlrp4*^{-/-} and *Aim2*^{-/-} mice were left uninfected (UI) or infected with *P. multocida* at an MOI of 1. After a 24 h incubation, the supernatants and cell lysates were collected. The amounts of IL-1β (A), IL-6 (B) and TNFα (C) in the supernatants were determined by ELISA. The culture supernatants and cell lysates were subjected to Western blot analysis (D). β-actin was detected as a control. Lysates, cell lysate; and Sup, supernatant. Data are representative of at least three independent experiments. Statistical significance was determined by Student's *t*-test (**P < 0.01).

macrophages infected by *P. multocida*, cytochalasin B was used to pre-treat macrophages to inhibit phagocytosis. The impaired engulfment of bacteria in macrophages pretreated with cytochalasin B resulted in a significant decrease in IL-1β secretion. Considering that cytochalasin B can also inhibit the secretion of eATP, which is a very common second

signal for the activation of the NLRP3 inflammasome, P2X7 receptor inhibitor (A438079) was used and the results showed that the IL-1β secretion is not affected by A438079 (Fig. 4C). These data indicated that the phagocytosis of *P. multocida* by macrophages plays an important role in IL-1β secretion.

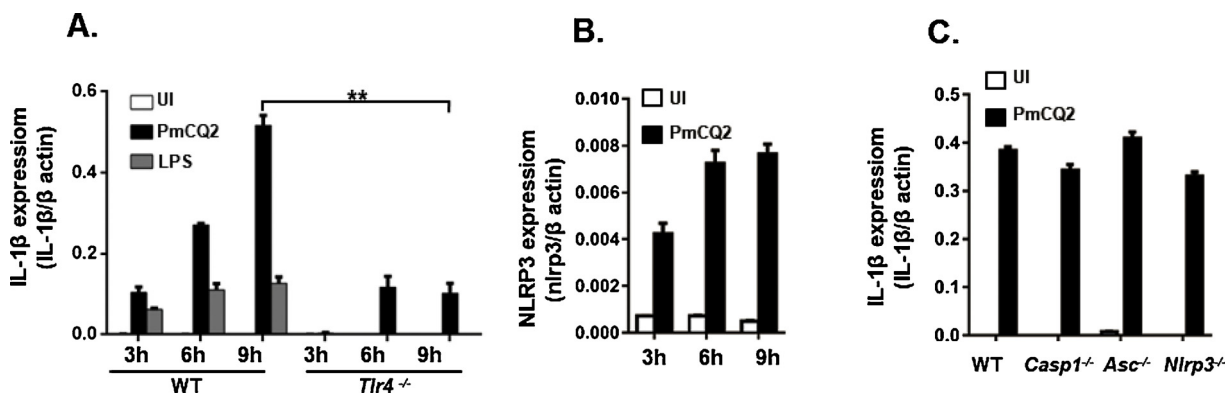


Fig. 3. NLRP3 inflammasome has no impact on IL-1β gene expression.

PECs from C57BL/6 WT or *Tlr4*^{-/-} mice were infected with *P. multocida* at an MOI of 1 or stimulated with LPS (50 ng/ml), and the total cellular RNA was extracted at the indicated times. The levels of IL-1β(A) and NLRP3 (B) mRNA expression were analyzed by real-time RT-PCR. PECs from C57BL/6 WT, *Casp1*^{-/-}, *Asc*^{-/-} and *Nlrp3*^{-/-} mice were infected with *P. multocida* at an MOI of 1 for 3, 6, or 9 h, and the expression of IL-1β was quantified by real-time RT-PCR (C). All of the experiments were repeated three or more times.

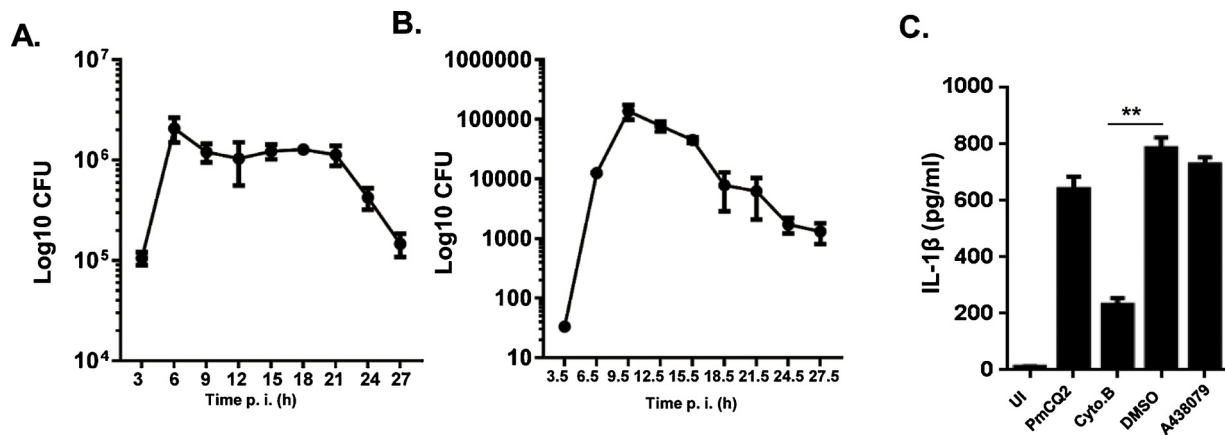


Fig. 4. Phagocytosis of *P. multocida* by macrophages is important for IL-1 β secretion.

Adherent PECs were infected with *P. multocida* at an MOI of 1. (A) The bacteria associated with the macrophages were counted. The infected macrophages were washed with chilled PBS three times and lysed with Triton X-100 at the indicated time points. The lysates were plated on Martin's agar plates after being serially diluted, and CFUs were counted after an overnight incubation. (B) The phagocytosed bacteria were counted. Non-cell-associated bacteria were removed by washing every 3 h until 27 h postinfection, and the macrophages were cultured for an additional 30 min in the presence of 100 mg/ml ciprofloxacin. The bacteria in the cell lysates were then counted. (C) The PECs were left untreated or pretreated with cytochalasin B, A438079 or DMSO and then infected with *P. multocida*. The supernatants were collected at 24 h postinfection. The levels of IL-1 β in the supernatants were determined by ELISA. The results are representative of three independent experiments. Statistical significance was determined by Student's *t*-test (***P* < 0.01).

3.5. Syk signaling pathway is involved in IL-1 β secretion in macrophages infected with *P. multocida*

The NLRP3 inflammasome responds to a wide range of PAMPs and DAMPs and has been extensively investigated. Instead of directly interacting with various stimuli, the NLRP3 inflammasome is activated through common cellular events. Some kinases have been reported to be upstream of NLRP3 and to be involved in NLRP3 activation. Syk has been reported to modulate the NLRP3 inflammasome activation in response to several infectious pathogens, it can induce ROS production or play roles through various downstream adaptor molecules and MAPK kinases (Gross et al., 2009; Lee et al., 2016; Lin et al., 2013). Hara et al. reported that Syk- and JNK-mediated ASC phosphorylation function as a molecular 'switch' that controls the formation of ASC specks and inflammasome activation (Hara et al., 2013). To further investigate the mechanism of NLRP3 inflammasome activation and subsequent IL-1 β secretion upon *P. multocida* infection, we pretreated the macrophages with several kinase inhibitors before the infection. The levels of IL-1 β in the culture supernatants were detected, and the results showed that there was a significant decrease in IL-1 β secretion in the BAY, Z-VAD and R406 treatment groups compared with the control group, but there were no significant differences in either the SB or SP treatment group compared with the untreated macrophage group (Fig. 5A–B). Furthermore, the activation of Syk and JNK upon *P. multocida* infection was assessed by detecting phosphorylated Syk or JNK, and the p-Syk or p-JNK was inhibited by the inhibitor R406 and SP, respectively (Fig. 5C–D). Although these kinases were induced in an infection-dependent manner, our results indicated that Syk, but not JNK or p38 MAPK, plays a vital role in the secretion of IL-1 β in *P. multocida*-infected macrophages.

4. Discussion

Innate immune responses play important roles in host defense against bacterial infection. In this study, we elucidated the mechanism of caspase-1 activation and IL-1 β secretion in macrophages infected with *P. multocida*. We found that the NLRP3 inflammasome, but not the AIM2 or NLRC4 inflammasome, was indispensable for inducing the activation of caspase-1 and the maturation and secretion of IL-1 β . This study revealed a role for the NLRP3 inflammasome in mediating the host proinflammatory response to *P. multocida*.

Inflammasomes are cytosolic multiprotein complexes that assemble to detect various stimuli and initiate rapid innate responses; recognition by inflammasomes has been reported for many bacterial pathogens (Sauer et al., 2011; Shin and Brodsky, 2015; Witzenth et al., 2011). Most of the reported bacteria are associated with human disease, while livestock-associated bacterial pathogens have been neglected. Several inflammasomes, including the NLRP1, NLRP3, NLRC4, AIM2 and non-canonical caspase-11 inflammasomes, can sense a specific bacterium or a series of related bacteria to induce host immune responses that benefit or damage the host (Moltke et al., 2013). Our study reported that in response to an infection with the bovine pathogen *P. multocida*, the NLRP3 inflammasome, but not the AIM2 or NLRC4 inflammasome, is assembled, and caspase-1 is activated in a macrophage infection model in vitro. However, how the NLRP3 inflammasome plays a role in *P. multocida*-host interactions remains unclear. Further in vivo studies using inflammasome component knockout mice infected with *P. multocida* should be carried out in the future.

The inflammasome is a platform for caspase-1 activation, and caspase-1 was first identified as an IL-1 β -converting enzyme. However, caspase-1 is not the only enzyme that cleaves IL-1 β into its mature form; serine protease granzyme A neutrophil proteinase 3 (PR3), neutrophil elastase (NE) and cathepsin G also have similar functions that mediate pro-IL-1 β processing (Alfaidi et al., 2015; Coeshott et al., 1999; Guma et al., 2009; Hildebrand et al., 2014). Hildebrand et al. reported that *Pasteurella multocida* toxin (PMT), which is a major virulence protein produced by some of the serotype A and D strains, induces granzyme A-mediated processing of IL-1 β independent of caspase-1 and the inflammasome (Hildebrand et al., 2014). In our study, the PmCQ2 strain did not contain PMT, and we demonstrated that NLRP3 inflammasome-induced caspase-1 activation is responsible for IL-1 β maturation and secretion. It is of interest to know whether other *P. multocida* serotype strains that do not produce PMT also require the NLRP3 inflammasome and caspase-1 for IL-1 β maturation.

Although *P. multocida* is not considered an intracellular bacterium, some previous reports have shown that *P. multocida* can invade a variety of cell types, such as primary turkey kidney cells and bovine endothelial cells (Al-haj Ali et al., 2004; Galdiero et al., 2001; Lee et al., 1994). *P. multocida* can be killed by turkey or mouse macrophages, which is consistent with the results of our study. However, other studies have reported that *P. multocida* can survive inside mouse macrophages (Collins et al., 1983). These differences might be due to the variation in

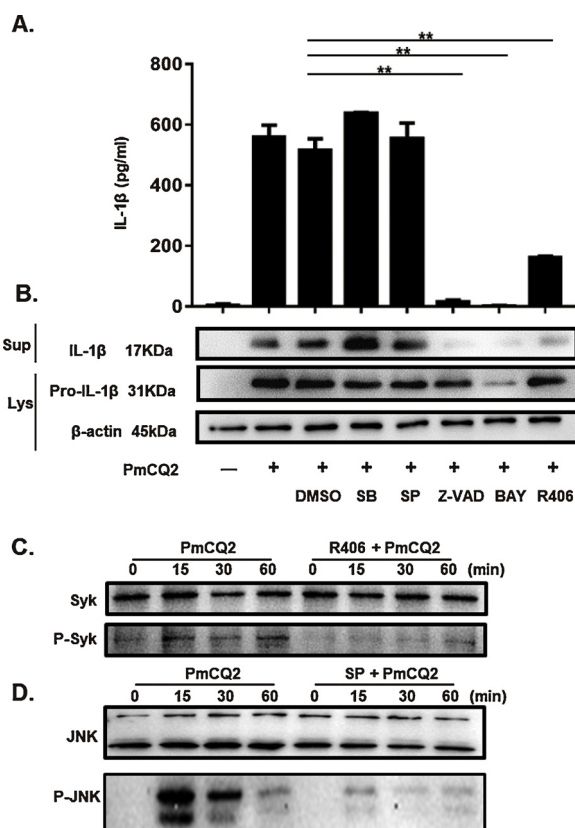


Fig. 5. Syk signaling is involved in IL-1 β maturation and secretion. PECs from C57BL/6 WT mice were left uninfected (UI) or infected with *P. multocida* at an MOI of 1. Kinase inhibitors were added to the cultures 1 h before the infection (A–D). After incubation, the supernatants and cell lysates were collected. The level of IL-1 β was determined by ELISA (A). The culture supernatants and cell lysates were subjected to Western blot analysis. β -actin was detected as a control. Lys, cell lysate; and Sup, supernatant (B). PECs infected with *P. multocida* at an MOI of 1 for 0, 15, 30 or 60 min. The cell lysates were collected, and total phosphorylated Syk and JNK levels were detected by western blotting (C–D). The targets and concentrations of the kinase inhibitors are as follows: SB (p38 inhibitor, 1 μ M), SP (JNK inhibitor, 10 μ M), BAY (NF- κ B inhibitor, 20 μ M), Z-VAD (caspase inhibitor, 10 μ M) and R406 (Syk inhibitor, 1 μ M). Data are representative of at least three independent experiments. Statistical significance was determined by Student's *t*-test (***P* < 0.01).

the serotypes and virulence of the strains used in the studies. However, in our study, we showed that the phagocytosis of *P. multocida* was required for IL-1 β secretion; phagocytosis of bacteria may be required for NLRP3 inflammasome activation. Other studies have provided evidence that the entry of *P. multocida* into macrophages is important for the induction of the immune response (Othman et al., 2012; Wilkie et al., 2012).

We have developed a mechanistic model of caspase-1 activation and IL-1 β maturation in macrophages infected by *P. multocida*. The NLRP3 inflammasome is critically involved in this process. Our results revealed a pathophysiological link between *P. multocida* and the NLRP3 inflammasome.

Conflict of interest statement

There is no conflict of interest in this manuscript.

Acknowledgements

We thank Dr. Feng Shao from NIBS (National Institute of Biological Sciences, Beijing) to provide the *Casp1*^{-/-}, *Asc*^{-/-}, *Nlrp3*^{-/-}, *Nlr4*^{-/-}

, *Aim2*^{-/-} and *Tlr4*^{-/-} mice. This study was supported by the National Key Research and Development Program of China (2018YFD0500500), the earmarked fund for China Agriculture Research System (CARS-37), the Chongqing Science & Technology Commission (cstc2015jcyjBX0108, cstc2017shms-zdyfx0036, cstc2018jscx-msybX0302), the Fundamental Research Funds for the Central Universities (XDJK2017A003) and the Innovation Team Building Program in Chongqing universities (CXTD201602004). The funding bodies had no role in study design, data collection or analysis, decision to publish or preparation.

References

- Alfaidi, M., Wilson, H., Daigneault, M., Burnett, A., Ridger, V., Chamberlain, J., Francis, S., 2015. Neutrophil elastase promotes Interleukin-1 β secretion from human coronary endothelium. *J. Biol. Chem.* 290, 24067–24078.
- Al-haj Ali, H., Sawada, T., Hatakeyama, H., Katayama, Y., Ohtsuki, N., Itoh, O., 2004. Invasion of chicken embryo fibroblast cells by avian *Pasteurella multocida*. *Vet. Microbiol.* 104, 55–62.
- Bauernfeind, F., Horvath, G., Stutz, A., Alnemri, E.S., Macdonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., 2009. NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* 183, 787–791.
- Borthwick, L.A., 2016. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Semin. Immunopathol.* 38, 517–534.
- Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S., Wiecek, M., Kroona, H., Leimer, A.H., Cheronis, J., 1999. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6261–6266.
- Collins, F.M., Niederbuhl, C.J., Campbell, S.G., 1983. Bactericidal activity of alveolar and peritoneal macrophages exposed in vitro to three strains of *Pasteurella multocida*. *Infect. Immun.* 39, 779–784.
- Dabo, S.M., Taylor, J.D., Confer, A.W., 2007. *Pasteurella multocida* and bovine respiratory disease. *Anim. Health Res. Rev.* 8, 129–150.
- Du, H., Fang, R., Pan, T., Li, T., Li, N., He, Q., Wu, R., Peng, Y., Zhou, Z., 2016. 1 Comparative genomics analysis of two different virulent bovine 2 *Pasteurella multocida* isolates. *Int. J. Genomics* 2016 (2016-12-14), 1–14 2016.
- Galdiero, M., De Martino, L., Pagnini, U., Pisciotto, M.G., Galdiero, E., 2001. Interactions between bovine endothelial cells and *Pasteurella multocida*: association and invasion. *Res. Microbiol.* 152, 57–65.
- Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hanneschläger, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459, 433.
- Guma, M., Ronacher, L., Ru, L.B., Takai, S., Karin, M., Corr, M., 2009. Caspase-1 independent IL-1 β activation in neutrophil dependent inflammation. *Arthritis Rheum.* 60, 3642–3650.
- Hara, H., Tsuchiya, K., Kawamura, I., Fang, R., Hernandez-Cuellar, E., Shen, Y., Mizuguchi, J., Schweighoffer, E., Tybulewicz, V., Mitsuyama, M., 2013. Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat. Immunol.* 14, 1247.
- Hildebrand, D., Bode, K.A., Riess, D., Cerny, D., Waldhuber, A., Rommler, F., Strack, J., Korten, S., Orth, J.H., Miethke, T., Heeg, K., Kubatzky, K.F., 2014. Granzyme A produces bioactive IL-1beta through a nonapoptotic inflammasome-independent pathway. *Cell Rep.* 9, 910–917.
- Hodgson, J.C., Dagleish, M.P., Gibbard, L., Bayne, C.W., Finlayson, J., Moon, G.M., Nath, M., 2013. Seven strains of mice as potential models of bovine pasteurellosis following intranasal challenge with a bovine pneumonic strain of *Pasteurella multocida* A:3; comparisons of disease and pathological outcomes. *Res. Vet. Sci.* 94, 634–640.
- Jo, E.K., Kim, J.K., Shin, D.M., Sasakawa, C., 2016. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell. Mol. Immunol.* 13, 148–159.
- Kharb, S., Charan, S., 2013. Mouse model of haemorrhagic septicaemia: dissemination and multiplication of *Pasteurella multocida* B:2 in vital organs after intranasal and subcutaneous challenge in mice. *Vet. Res. Commun.* 37, 59–63.
- Lee, M.D., Wooley, R.E., Glisson, J.R., 1994. Invasion of epithelial cell monolayers by turkey strains of *Pasteurella multocida*. *Avian Dis.* 38, 72–77.
- Lee, C.K., Yang, Y., Chen, C., Liu, J., 2016. Syk-mediated tyrosine phosphorylation of Mule promotes TNF-induced JNK activation and cell death. *Oncogene* 35, 1988.
- Lin, Y.C., Huang, D.Y., Chu, C.L., Lin, Y.L., Lin, W.W., 2013. The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Sci. Signal.* 6 ra71.
- Manoranjani Sahoo, I.C.-O., Laura del Barrio, Re, Fabio, 2011. Role of the inflammasome, IL-1 β , and IL-18 in bacterial infections. *Sci. World J.* 11, 2037–2050.
- Moltke, Jv., Ayres, J.S., Kofoed, E.M., ChavarríaSmith, J., Vance, R.E., 2013. Recognition of Bacteria by inflammasomes. *Annu. Rev. Immunol.* 31, 73–106.
- Othman, S., Parton, R., Coote, J., 2012. Interaction between mammalian cells and *Pasteurella multocida* B:2. Adherence, invasion and intracellular survival. *Microb. Pathog.* 52, 353–358.
- Pors, S.E., Chadfield, M.S., Sorensen, D.B., Offenberg, H., Bisgaard, M., Jensen, H.E., 2016. The origin of *Pasteurella multocida* impacts pathology and inflammation when assessed in a mouse model. *Res. Vet. Sci.* 105, 139–142.

- Praveena, P.E., Periasamy, S., Kumar, A.A., Singh, N., 2010. Cytokine profiles, apoptosis and pathology of experimental *Pasteurella multocida* serotype A1 infection in mice. *Res. Vet. Sci.* 89, 332–339.
- Sansonetti, P.J., Phalipon, A., Arondel, J., Thirumalai, K., Banerjee, S., Akira, S., Takeda, K., Zychlinsky, A., 2000. Caspase-1 activation of IL-1 β and IL-18 are essential for *Shigella flexneri*-induced inflammation. *Immunity* 12, 581–590.
- Sauer, J.D., Pereyre, S., Archer, K.A., Burke, T.P., Hanson, B., Lauer, P., Portnoy, D.A., 2011. *Listeria monocytogenes* engineered to activate the Nlr4 inflammasome are severely attenuated and are poor inducers of protective immunity. *Proc. Natl. Acad. Sci. U. S. A.* 108, 12419–12424.
- Shin, S., Brodsky, I.E., 2015. The inflammasome: learning from bacterial evasion strategies. *Semin. Immunol.* 27, 102–110.
- Wen, H., Miao, E.A., Ting, J.P., 2013. Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity* 39, 432–441.
- Wilkie, I.W., Harper, M., Boyce, J.D., Adler, B., 2012. *Pasteurella multocida*: diseases and pathogenesis. *Curr. Top. Microbiol. Immunol.* 361, 1–22.
- Witzenrath, M., Pache, F., Lorenz, D., Koppe, U., Gutbier, B., Tabeling, C., Reppe, K., Meixenberger, K., Dorhoi, A., Ma, J., 2011. The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J. Immunol.* 187, 434–440.
- Wu, C., Qin, X., Li, P., Pan, T., Ren, W., Li, N., Peng, Y., 2017. Transcriptomic analysis on responses of murine lungs to *Pasteurella multocida* Infection. *Front. Cell. Infect. Microbiol.* 7, 251.