Molecular Immunology 116 (2019) 11–17

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# Critical roles of NLRP3 inflammasome in IL-1β secretion induced by *Corynebacterium pseudotuberculosis* in vitro

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ARTICLE INFO

*Keywords:*
*Corynebacterium pseudotuberculosis*
Interleukin-1β
NLRP3 inflammasome
TLR4
NF-κB
p38MAPK

ABSTRACT

*Corynebacterium pseudotuberculosis* is a prominent human and animal pathogen causing chronic inflammatory diseases. Interleukin-1β (IL-1β) is involved in the response to such pathogenic infections. However, the mechanism by which IL-1β is secreted during *C. pseudotuberculosis* infection remains unclear. This study aimed to investigate the mechanism underlying IL-1β secretion by macrophages infected with *C. pseudotuberculosis*. Herein, we firstly revealed that nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 (Casp1) play critical roles in IL-1β secretion rather than IL-1β precursor (pro-IL-1β) expression in *C. pseudotuberculosis*-infected macrophages. Toll like receptor 4 (TLR4) is partially involved in IL-1β secretion, while absent in melanoma 2 (AIM2) is not involved in IL-1β secretion by *C. pseudotuberculosis*-infected macrophages. In addition, nuclear factor kappa B (NF-κB) and p38 mitogen-activated protein kinases (p38 MAPK) inhibitors almost attenuated IL-1β secretion, implying that NF-κB and p38MAPK pathway are involved in IL-1β secretion in *C. pseudotuberculosis*-infected macrophages. Furthermore, *C. pseudotuberculosis* were significantly more numerous in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp*-1$^{-/-}$ macrophages than in WT macrophages at 24 h after infection ($P < 0.05$), indicating that NLRP3 inflammasome components limit *C. pseudotuberculosis* replication in macrophages. Together, these data provide novel insights into the mechanisms underlying IL-1β secretion in *C. pseudotuberculosis*-infected macrophages and further the current understanding of the host pro-inflammatory immune response against this pathogen.

## 1. Introduction

*Corynebacterium pseudotuberculosis* is a gram-positive and facultative intracellular pathogen causing multiple chronic diseases including caseous lymphadenitis in sheep and goats, bovine mastitis, ulcerative lymphangitis in horses and necrotizing lymphadenitis in humans (Barauna et al., 2017; Silva et al., 2011; Trost et al., 2010; Viana et al., 2017). Since *C. pseudotuberculosis* has a long duration in the external environment, subclinically infected animals are not easy to detect, and pharmacotherapy after infection is not satisfactory, once this pathogen is introduced into the herd animal, it is extremely difficult to control and eradicate (Dorella et al., 2006). Therefore, a better understanding of the host immune response to *C. pseudotuberculosis* infections will help develop approaches to control this disease.

Innate immune system is extremely important during pathogenic infections. Inflammation, prominent manifestation of innate immunity, is the primary host defense; however, aberrant inflammation is hazardous to the host (Dinarello, 2018). *C. pseudotuberculosis* infections are characterized by the formation of pyogranuloma and abscesses in internal organs and external lymph nodes, wherein inflammatory cytokines are significantly upregulated (Pepin et al., 1997). Interleukin-1β (IL-1β), one of the most important inflammatory cytokines in IL-1 superfamily, is a key mediator of the acute-phase of inflammation and plays important roles in response to pathogenic infections (Dinarello,

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https://doi.org/10.1016/j.molimm.2019.09.016
Received 29 June 2019; Received in revised form 1 August 2019; Accepted 14 September 2019
Available online 25 September 2019

2009; Slaats et al., 2016). IL-1β is reportedly upregulated in sheep with pyogranulomas in the draining lymph node compared to those without pyogranulomas after *C. pseudotuberculosis* infection (Pepin et al., 1997), indicating that IL-1β is essential for regulating the response to this pathogen. However, the mechanism underlying *C. pseudotuberculosis*-mediated activation of IL-1β secretion is unclear.

Two signals are speculated to be involved in the IL-1β secretion: signal I, called priming, induces NF-κB-dependent expression of both IL-1β precursor (pro-IL-1β) and inflammasome after pattern-recognition receptors (PRRs), and signal II triggers inflammasome assembly and activation (Liu et al., 2018). Inflammasome activation causes cleavage of the pro-IL-1β to mature IL-1β by active caspase-1 (Liu et al., 2018); thereafter, mature IL-1β is secreted through membrane pores via activated gasdermin D (Liu et al., 2016; Shi et al., 2015). This study aimed to investigate the mechanism underlying IL-1β secretion by macrophages infected with *C. pseudotuberculosis*, and the present results may help understand the host pro-inflammatory immune response against this pathogen.

## 2. Material and methods

### 2.1. Reagents

Thioglycolate medium was purchased from Eiken (Japan). Brain Heart Infusion (BHI) broth was obtained from Hangzhou Binhe Microorganism Reagent Co., Ltd. (China). Fosfomycin was obtained from National Institutes for Food and Drug Control (China). RPMI -1640 and fetal bovine serum (FBS) were obtained from Biological Industries (Israel). Opti-MEM was purchased from Gibco (USA). IL-1β, IL-6 and TNF-α ELISA kits, radioimmunoprecipitation assay (RIPA) lysis buffer and Protein Marker were purchased from Invitrogen/Thermo Fisher Scientific (USA). Mouse anti-IL-1β antibody was obtained from R &D Systems (Minneapolis, USA). Anti-β-actin monoclonal antibody was obtained from Proteintech (USA). HRP-labeled rabbit anti-goat IgG and HRP-goat anti-mouse IgG were obtained from ZSGB-BIO (China). BAY11-7082 (NF-κB inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) and LY294002 (PI3K inhibitor), BeyoECL Star and Glycine were obtained from Beyotime (China). R406 (Syk inhibitor) was purchased from Selleckchem (USA). Gentamicin, nalidixic acid and Triton-100 were obtained from Solarbio (China).

### 2.2. Mice

C57BL/6 mice were purchased from the Chongqing Academy of Chinese Materiel Medical, Chongqing, China (permit No. SCXK(Yu) 2012-0001). Toll-like receptor 4-knockout (*Tlr4*$^{-/-}$), AIM2-knockout (*Aim2*$^{-/-}$), NLRP3-knockout (*Nlrp3*$^{-/-}$), ASC-knockout (*Asc*$^{-/-}$), and Caspase-1-knockout (*Casp-1*$^{-/-}$) C57BL/6 mice were kindly provided by Feng Shao from the NIBS (National Institute of Biological Sciences, Beijing, China). The mice were housed at 24 ± 2℃ and a relative humidity of 55 ± 15% in clean cages and fed with standard rodent diet; they were used at 8 to 10 weeks of age. All animal experiments were approved by the Ethics Committee of Southwest University, Chongqing, China.

### 2.3. Bacterial culturing

*C. pseudotuberculosis* XH02 strain was isolated from a Boer Goat in Xuanhan, China (Zhou et al., 2016) and stored in −80℃. *C. pseudotuberculosis* were cultured at 37℃ in BHI broth (containing 5% FBS, 200 μg/ml fosfomycin and 4 μg/ml nalidixic acid) (Zhao et al., 1991) for 24 h with agitation at 180 rpm (rpm). Bacterial density was determined through a viable count on agar plates containing 5% rabbit blood.

### 2.4. Extraction of macrophages

Peritoneal macrophages were obtained as reported previously (Fang et al., 2017). Briefly, peritoneal exudate cells (PECs) were harvested from the peritoneal lavage of mice intraperitoneally injected with 3 ml of thioglycolate medium 3 d prior, and PECs were washed and suspended in RPMI 1640 supplemented with 10% FBS and 100 μg/ml gentamicin, and incubated in 48-well microplates ($2.5 \times 10^5$ cell/well) at 37 ˚C with 5% $CO_2$. Two hours after incubation, non adherent cells eliminated via three washes with PBS, and adherent cells were used as peritoneal macrophages.

### 2.5. ELISA

Adherent peritoneal macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS, and infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added to the cultures (final concentration, 100 μg/ml), and the culture supernatants and cell lysates were collected after an additional 22 h. Culture supernatants of relevant assays were collected and stored at -20℃ prior to quantification of cytokines. Levels of IL-1β, IL-6 and TNF-α in culture supernatants were determined via a two-site sandwich ELISA n accordance with the manufacturer's instructions.

### 2.6. Quantitative RT-PCR analysis

Adherent peritoneal macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS and infected with *C. pseudotuberculosis* at MOI of 10 for 2 h, and gentamicin was added to cultures (final concentration, 100 μg/ml) for 2 h. Total RNA was extracted with RNAiso Plus, and cDNA was synthesized with PrimeScript RT Reagent Kit. Target gene mRNA expression was assessed via real-time PCR analysis using TB Green Premix Ex Taq II with the following primers: *IL-1 β* SF: AACGTGTGGGGGATGAATTG, SR: CATACTCATCAAAGCAA TGT; *TNF-α* SF: CTCCAGCTGGAAGACTCCTCCCAG, SR: CCCGACTAC GTGCTCCTCACC; *Nlrp3* SF: ATGGCTGTGTGGATCTTTGC, SR: CACGT GTCATTCCACTCTGG; *Casp 1* SF: CCAGGCAAGCCAAATCTTTA, SR: TCAGCTGATGGAGCTGATTG; *β-actin* SF: CTAAGGCCAACCGTGAA AAG, SR: ACCAGAGGCATACAGGG- ACA. The expression of above genes was analyzed using the $2^{-\Delta\Delta Ct}$ method with β-actin as the endogenous housekeeping gene.

### 2.7. Western blot analysis

Peritoneal macrophages ($5 \times 10^5$ cell/well), cultured in 12-well plates with Opti-MEM, were infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added in the medium (final concentration, 100 μg/ml). Culture supernatants were harvested after 22 h, and the cells were lysed with RIPA buffer. Proteins in the supernatants and cell lysates were separated via SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The membranes were probed with anti-IL-1β or anti-β actin antibody and the corresponding HRP-labeled secondary antibody for 1 h, and the signal was detected with BeyoECL Star kit.

### 2.8. Quantification of C. pseudotuberculosis replication in macrophages

Peritoneal macrophages ($1 \times 10^5$ cell/well), derived from WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *and Casp-1*$^{-/-}$ C57BL/6 mice were cultured in a 24-well plate in RPMI 1640 medium and infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, gentamicin was added to the culture medium (final concentration, 100 μg/ml). Macrophages were treated with 0.5% Triton X-100 at 2, 8, 16, and 24 h after infection, and the total number of bacteria in macrophages were determined via the plate dilution counting method.

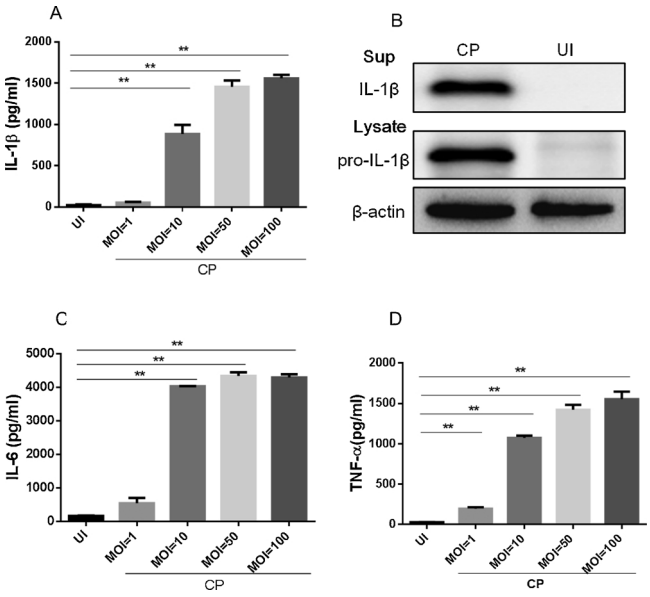


**Fig. 1.** *Corynebacterium pseudotuberculosis* infection induced IL-1β, IL-6, and TNF-α secretion in macrophages. Peritoneal macrophages from C57BL/6 wild-type (WT) mice were infected with *C. pseudotuberculosis* at an MOI of 1, 10, 50, and 100 for 2 h, and gentamicin was added to the culture media (final concentration, 100 μg/ml). Culture supernatants and cell lysates were harvested after an additional 22 h. Levels of IL-1β (A), IL-6 (C), and TNF-α (D) were determined via ELISA. Expression of pro-IL-1β in cell lysates and mature IL-1β in supernatants after infection (MOI = 10) were detected via western blot analysis (B). Data are presented as mean ± SEM values and are representative of three independent experiments. CP: *C. pseudotuberculosis*; Lysate: cell lysate; Sup: supernatants; UI: uninfected. $^{**}P < 0.01$compared with UI group.

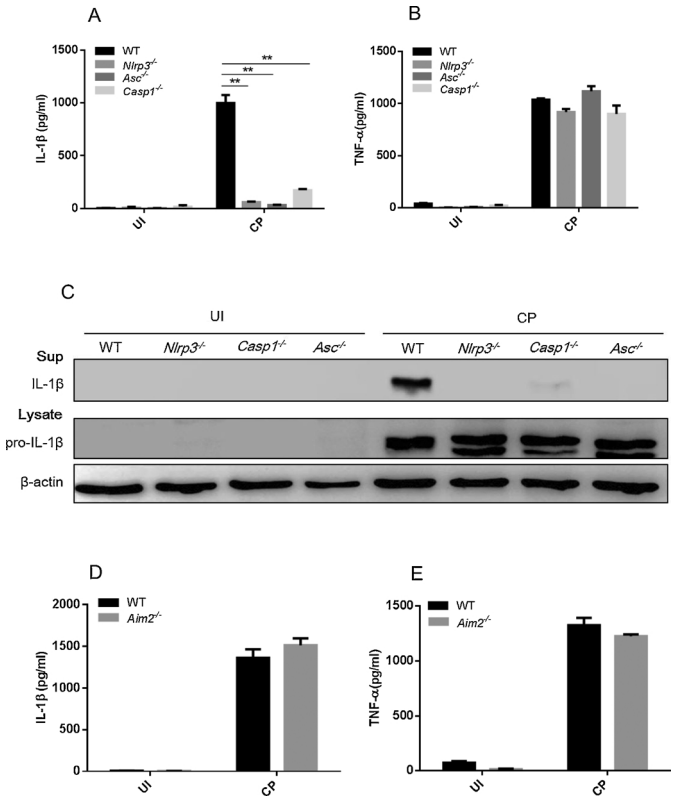


**Fig. 2.** Secretion of IL-1β in macrophages infected with *Corynebacterium pseudotuberculosis* is dependent on NLRP3, ASC, and Casp-1. Peritoneal macrophages from C57BL/6 WT, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *and Casp 1*$^{-/-}$ mice were infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added to the culture media (final concentration, 100 μg/ml). The culture supernatants and cell lysates were harvested after an additional 22 h. The levels of IL-1β (A, D) and TNF-α (B, E) in the culture supernatants were detected via ELISA. Expression of pro-IL-1β in cell lysates and mature IL-1β in supernatants were detected via western blot analysis (C). Data are presented as mean ± SEM values and are representative of three independent experiments. CP: *C. pseudotuberculosis*; Lysate: cell lysate; Sup: supernatants; UI: uninfected; $^{**}P < 0.01$ compared with CP infected WT group.

### 2.9. Statistical analysis

The results were analyzed using GraphPad Prism 5.0 software and Student's *t* test. The results are reported as means ± standard error. *P*-values of < 0.05 were considered significant ($^{*}P < 0.05$ and $^{**}P < 0.01$).

## 3. Results

### 3.1. C. Pseudotuberculosis activates IL-1β, IL-6, and TNF-α secretion in macrophages

To determine whether *C. pseudotuberculosis* infection in macrophages cause IL-1β, IL-6, and TNF-α secretion, we assessed their expression levels in the supernatants of infected macrophages and pro-IL-1β in cell lysates. As shown in Fig. 1A–D, pro-IL-1β, IL-1β, IL-6, and TNF-α were significantly increased after *C. pseudotuberculosis* infection ($P < 0.01$). These results indicate that *C. pseudotuberculosis* infection activates a proinflammatory response in macrophages.

### 3.2. NLRP3, ASC, and Casp-1 play critical roles in C. pseudotuberculosis-induced IL-1β secretion

To verify the role of AIM2 and NLRP3 inflammasomes in IL-1β and TNF-α secretion after *C. pseudotuberculosis* infection, we assessed the levels of IL-1β and TNF-α in the supernatants of infected macrophages from WT, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp-1*$^{-/-}$ mice, and the expression of pro-IL-1β in cell lysates. IL-1β secretion was significantly abrogated in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Casp-1*$^{-/-}$ macrophages compared with WT macrophages ($P < 0.01$) (Fig. 2A), while TNF-α levels, independent of inflammasomes, showed no significant difference in each group (Fig. 2B). Western blot analysis confirmed the critical role of *Nlrp3*, *Asc*, and *Casp-1* in IL-1β secretion in *C. pseudotuberculosis*-infected macrophages; however, pro-IL-1β expression was not significantly affected in the absence of the aforementioned genes (Fig. 2C). No significant difference in IL-1β and TNF-α secretion were observed in *C. pseudotuberculosis*-infected macrophages from WT and *Aim2*$^{-/-}$ mice (Fig. 2D–E). Together, these data indicate that NLRP3 inflammasomes play key roles in IL-1β secretion, albeit dispensable for pro-IL-1β expression, and AIM2 is not involved in IL-1β secretion in macrophages infected with *C. pseudotuberculosis*.

### 3.3. TLR4 is partially involved in IL-1β secretion by macrophages infected with C. pseudotuberculosis

To determine the role of TLR4 in IL-1β and TNF-α secretion upon *C. pseudotuberculosis* infection, we determined IL-1β and TNF-αin the supernatants and the pro-IL-1β levels and *IL-1β*, *TNF-α*, *Nlrp3,* and *Casp-1* mRNA expression levels in infected macrophages from wild-type (WT) and *Tlr4*$^{-/-}$ mice. Consequently, IL-1β and TNF-α secretion was significantly lower in *C. pseudotuberculosis*-infected *Tlr4*$^{-/-}$ macrophages than in infected WT macrophages ($P < 0.05$) (Fig. 3A–C); However, the levels of these two cytokines in *C. pseudotuberculosis*-infected *Tlr4*$^{-/-}$ macrophages were significantly higher than those of uninfected cells (Fig. 3A–B), while no significant difference in pro-IL-1β and *IL-1β* mRNA expression levels were observed in the absence of *Tlr4* (Fig. 3C–D). *C. pseudotuberculosis* infection significantly upregulated

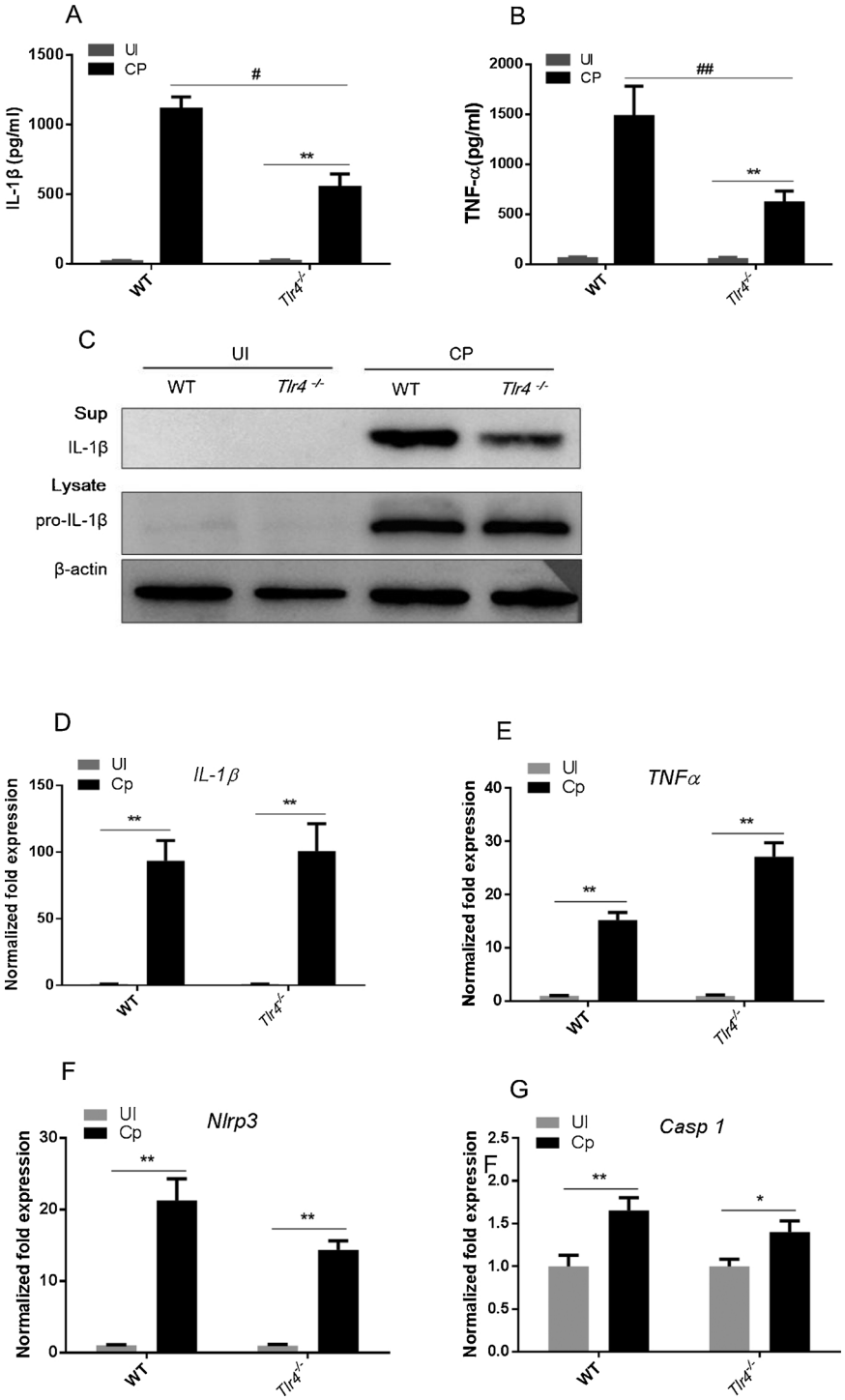


**Fig. 3.** TLR4 is partially involved in IL-1β secretion in macrophages infected with *Corynebacterium pseudotuberculosis*. Peritoneal macrophages from C57BL/6 WT and *Tlr4*$^{-/-}$ mice were infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added to the culture media (final concentration, 100 μg/ml). Total RNA was extracted from the cells after an additional 2 h, and the culture supernatants and cell lysates were harvested after 22 h. The levels of IL-1β (A) and TNF-α (B) in the culture supernatants were determined via ELISA. The pro-IL-1β levels in cell lysates and mature IL-1β in supernatants were detected via western blot analysis (C). Levels of *IL-1β* (D), *TNF-α* (E), *Nlrp3* (F), and *Casp 1* (G) mRNA expression were determined via real-time PCR analysis. The data are presented as mean ± SEM values and are representative of three or two independent experiments (for mRNA expression). CP: *C. pseudotuberculosis*; $^{**}P < 0.01$ compared with UI group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with CP infected WT group.

*TNF-α*, *Nlrp3,* and *Casp-1* mRNA expression in WT and *Tlr4*$^{-/-}$ macrophages (Fig. 3E–G), while the *Nlrp3* expression levels were lower in *Tlr4*$^{-/-}$ macrophages (14.35 fold) than in WT macrophages (21.28 fold) (Fig. 3F). These results indicate that TLR4 is partially involved in IL-1β secretion in macrophages infected with *C. pseudotuberculosis*, probably because of the relative downregulation of *Nlrp3* in *Tlr4*$^{-/-}$ macrophages infected with *C. pseudotuberculosis*.

### 3.4. The NF-κB and p38MAPK signaling pathways are involved in IL-1β secretion in macrophages activated by C. pseudotuberculosis

To analyze the signals associated with IL-1β secretion in *C. pseudotuberculosis*-infected macrophages, the NF-κB, Syk, p38MAPK, PI3K, and JNK pathways were suppressed with relevant inhibitors. Inhibition of NF-κB almost completely abolished IL-1β secretion ($P < 0.01$), followed by treatment with a p38MAPK inhibitor, which downregulated IL-1β by approximately 50% (Fig. 4A). Concurrently, upon addition of NF-κB and p38MAPK inhibitors, pro-IL-1β and mature IL-1β expression were significantly abrogated in *C. pseudotuberculosis*-infected macrophages, as analyzed via western blotting (Fig. 4B). Similarly, TNF-α levels in macrophages after *C. pseudotuberculosis* infection were significantly reduced by NF-κB and p38MAPK inhibitors ($P < 0.01$) (Fig. 4C). These results suggest that the NF-κB and p38 MAPK signal pathways are involved in IL-1β secretion in macrophages

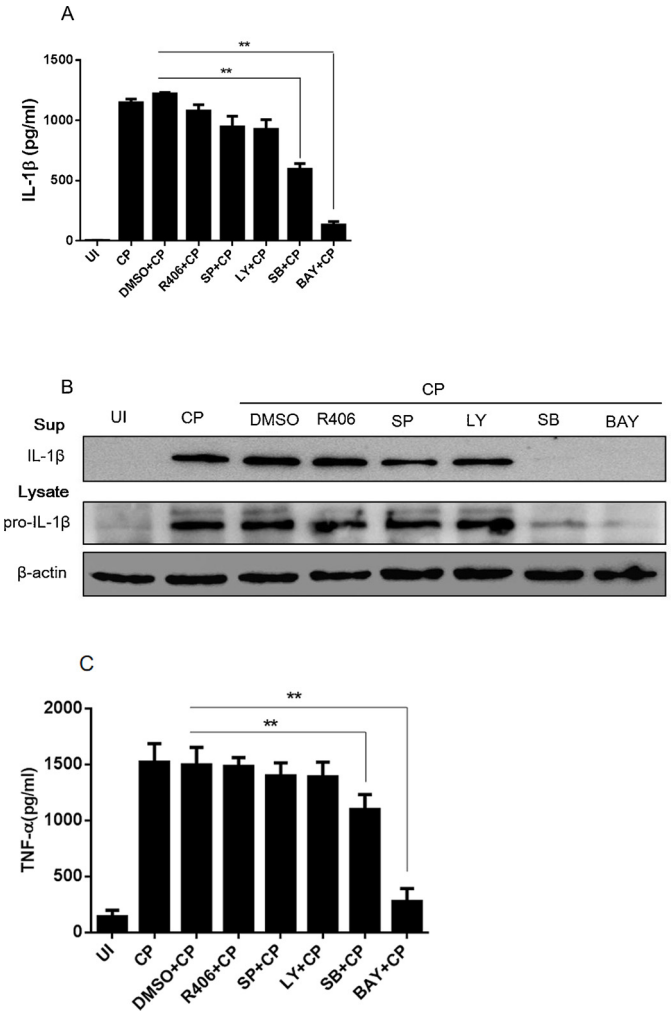


**Fig. 4.** The NF-κB and p38MAPK signaling pathways are involved in IL-1β secretion in macrophages infected with *Corynebacterium pseudotuberculosis*. Peritoneal macrophages were cultured in the presence of kinase inhibitors (R406 for Syk, 2 μM; SP600125/SP for JNK, 10 μM; LY294002/LY for PI3K, 200 nM; SB203580/SB for p38 MAPK, 30 μM; BAY 11-7082/BAY for NF-κB, 20 μM) for 1 h before infection. Thereafter, cells were infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added to the culture medium (final concentration, 100 μg/ml). The culture supernatants and cell lysates were harvested after an additional 22 h. The levels of IL-1β (A) and TNFα (C) in the culture supernatants were determined via ELISA. The levels of pro-IL-1β in lysates of cells and mature IL-1β in supernatants were detected via western blot analysis (B). Data are presented as mean ± SEM values and represent three independent experiments. CP: *C. pseudotuberculosis*; Lysate: cell lysate; Sup: supernatants; UI: uninfected. $^{**}P < 0.01$ compared with DMSO + CP treated macrophages.

infected with *C. pseudotuberculosis*.

### 3.5. NLRP3, ASC, and Caspase-1 limit C. pseudotuberculosis replication in macrophages

To determine the effect of the NLRP3 inflammasome on *C. pseudotuberculosis* replication after infection in macrophages, the number of *C. pseudotuberculosis* in macrophages from WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *and Casp-1*$^{-/-}$ mice was detected after infection at different timepoints. The total number of *C. pseudotuberculosis* in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp-1*$^{-/-}$ macrophages was significantly higher at 24 h after infection than that in WT macrophages ($P < 0.01$) (Fig. 5), suggesting that NLRP3, ASC, and Casp-1 limit *C. pseudotuberculosis* replication in macrophages.

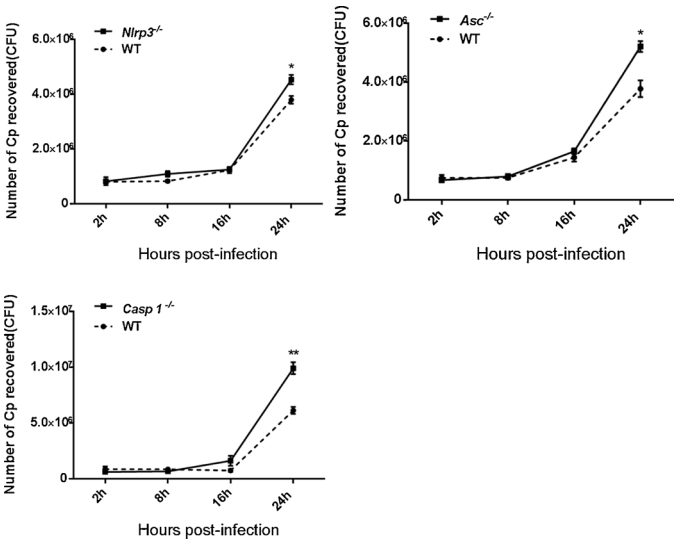


**Fig. 5.** NLRP3, ASC, and Caspase-1 limit *Corynebacterium pseudotuberculosis* replication in macrophages. Peritoneal macrophages from C57BL/6 WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *and Casp-1*$^{-/-}$ mice were infected with *C. pseudotuberculosis* at an MOI of 10 for 2 h, and gentamicin was added to the culture media (final concentration, 100 μg/ml). The number of bacteria recovered from macrophages (lysed by 0.5% Triton X-100) were determined via the plate dilution counting method at 2, 8, 16, and 24 h after infection. The data are presented as mean ± SEM values and represent two independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WT macrophages.

## 4. Discussion

An inflammatory response is the primary form of the host defense against bacterial infections. Upon infection by bacteria or other stimulus, macrophages could secret a series of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β etc. All these cytokines require the first signaling cascades that translocate NF-κB into the nucleus and induced transcription of these cytokines. But unlike TNF-α or IL-6, which is secreted directly after synthesis, the synthesis of IL-1β is existed as an inactive precursor protein, pro-IL-1β. The maturation and secretion of mature IL-1β requires a second signaling which is known as inflammasome assembling and caspase-1 activation. In this study, several inflammasome componenets knockout macrophages were used to test the involvement of the second signaling pathway in IL-1β maturation in macrophages infected with *C. pseudotuberculosis*. *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp-1*$^{-/-}$ macrophages showed defected IL-1β secretion, but not *Aim2*$^{-/-}$ macrophages. And in all the groups, secretion of TNF-α were not affected, which indicated that the first signaling cascades were not affected in these knockout macrophages. This study first reported the critical involvement of the NLRP3 inflammasomes in IL-1β secretion in macrophages infected with *C. pseudotuberculosis*.

Inflammasome assembly and activation is critical for IL-1β maturation and secretion. AIM2 and NLRP3 are the most widely characterized inflammasomes, both being activated in macrophages infected with *Listeria monocytogenes* (Kim et al., 2010). AIM2 is critical in controlling *Francisella tularensis* infections (Fernandes-Alnemri et al., 2010) and mediating host defense against *Mycobacterium tuberculosis* (Saiga et al., 2012). In contrast with a previous report that the deficiency of AIM2 severely impaired IL-1β secretion in *M. tuberculosis*-infected macrophages (Saiga et al., 2012), this study shows that AIM2 is not involved in IL-1β secretion, probably owing to the recognition of dsDNA of bacteria by AIM2 (Rathinam et al., 2010), and other than mycobacterial infections, *C. pseudotuberculosis* infections do not prevent the fusion of phagosomes and lysosomes but can resist lysosomal enzymes (Dorella et al., 2009), which may consequently not cause the release of DNA from *C. pseudotuberculosis* in macrophages.

NLRP3 inflammasomes can be activated by many infectious pathogens and mediates IL-1β secretion, such as *Streptococcus pneumonia* (Witzenrath et al., 2011), *Brucella abortus* (Gomes et al., 2013), *Yersinia pestis* (Zheng et al., 2011), *L. monocytogenes* (Meixenberger et al., 2010), Group B streptococcus infection (Costa et al., 2012), *Neospora caninum* (Wang et al., 2017), and *Pasteurella multocida* (Fang et al., 2019). The present results show that IL-1β secretion by macrophages infected with *C. pseudotuberculosis* depends on the components of the NLRP3 inflammasome, which limit *C. pseudotuberculosis* replication in macrophages, concurrent with previous reports indicating that mice lacking caspase-1 displayed higher levels of *Listeria* replication (Kim et al., 2010). However, the exact mechanism underlying *C. pseudotuberculosis*-mediated activation of NLRP3 inflammasomes is unclear. Furthermore, in vivo studies are required with the aforementioned gene knockout mice infected with *C. pseudotuberculosis*.

TLR4 primarily responds to LPS or endotoxins of gram-negative bacteria (Vijay, 2018), recognizes pneumolysin of *Streptococcus pneumoniae* (Malley et al., 2003) and Phenol-soluble modulins α1–α3 of *Staphylococcus aureus* (Chu et al., 2018), and mediate pneumolysin-induced ATF3 expression, which provides protection against pneumococcal infection by positively regulating cytokines (Nguyen et al., 2014, 2015). This study shows that IL-1β secretion is partially dependent on TLR4 in macrophages infected with *C. pseudotuberculosis*, and the reasons may be associated with the relative downregulation of *Nlrp3* mRNA expression in $Tlr4^{-/-}$ macrophages infected with *C. pseudotuberculosis*. However, the exact mechanism underlying the regulation of IL-1β secretion by TLR4 in *C. pseudotuberculosis*-infected macrophages warrants further investigation.

NF-κB and MAPKs are key factors/kinases that regulate multiple biological processes, including cell growth and differentiation and immune and inflammatory responses (Arthur and Ley, 2013; Liu et al., 2017). The activation states and models of NF-κB and MAPKs are not necessarily identical owing to differences in pathogens and host cell types. Herein, inhibition of p38MAPK and NF-κB significantly downregulated pro-IL-1β and almost completely abrogated IL-1β secretion, suggesting that the p38MAPK and NF-κB pathway are involved in IL-1β secretion in *C. pseudotuberculosis*-infected macrophages. Furthermore, this study indicates the involvement of these two pathways in TNF-α secretion upon *C. pseudotuberculosis* infection, concurrent with the results obtained with group B streptococci-infected monocytes (Mancuso et al., 2002). Further studies are required to elucidate the role of p38MAPK and NF-κB in the inflammatory response to a *C. pseudotuberculosis* infection.

In conclusion, this study shows that *C. pseudotuberculosis* induces IL-1β secretion in macrophages independent of AIM2 but in an NF-κB-, p38MAPK-, and NLRP3 inflammasome-dependent manner, being partially dependent on TLR4. Components of the NLRP3 inflammasome restrict *C. pseudotuberculosis* replication in macrophages. These findings elucidate the mechanism underlying IL-1β secretion in macrophages infected with *C. pseudotuberculosis*, thus furthering the current understanding of the host pro-inflammatory immune response against this pathogen.

## Declaration of Competing Interest

None of the authors have any conflict of interest.

## Acknowledgements

We thank Dr. Feng Shao from NIBS (National Institute of Biological Sciences, Beijing) to provide the $Casp1^{-/-}$, $Asc^{-/-}$, $Nlrp3^{-/-}$, $Tlr4^{-/-}$ and $Aim2^{-/-}$ mice. This study was supported by the National Key Research and Development Program of China (2018YFD0500500), Fundamental Research Funds for the Central Universities (XDJK2018C054) and in part by the Social Undertakings and Livelihood Security Technology Innovation Projects of Chongqing (CSTC2018JSCX-MSYBX0216) and the Plan of Entrepreneurial and Innovative Support for Overseas Students in Chongqing (CX2017103).

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