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Caspase-11 plays an important role in IL-1, IL-18 and IL-1β secretion from porcine alveolar macrophage cells stimulated with *Brucella suis* LPS

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Abstract

Brucella spp. is the causative agent of brucellosis, an extremely important disease worldwide. Innate immune cells detect pathogens via repeated cellular patterns (PAMPs) such as the *Brucella* suis (*B. suis*) lipopolysaccharide (LPS) coat on Gram-negative bacteria, which act as an important virulence factor of *Brucella*. *B.suis* LPS may be an issue for pro-inflammatory cytokine induction such as interleukin-1 (IL-1), interleukin-18 (IL-18) and interleukin-1β (IL-1β), which released from immune cells to mediate downstream inflammatory effects. To elucidate the mechanism of how *B.suis* LPS affects the secretion of IL-1, IL-18 and IL-1β in macrophages. We identified the up-regulation of caspase-11 in a porcine alveolar macrophages (PAM) cells stimulated with *B.suis* LPS. Furthermore, specific small interfering RNA (siRNA) targeting caspase-11 effectively inhibited *B.suis* LPS stimulated IL-1, IL-18 and IL-1β release from PAM. The results indicated that the concentrations of IL-1, IL-18 and IL-1β of caspase-11 siRNA pretreated group were lower than that of control significantly. Caspase-11 plays an important role in IL-1, IL-18 and IL-1β secretion from porcine alveolar macrophage cells stimulated with Brucella suis LPS and these findings might aid our understanding of the pathogenic mechanisms of *Brucella* and provide an entirely new innate immune response mechanism underlying macrophages dysfunction during *Brucella* infection.

Key words: *B.suis* LPS, Caspase-11, IL-1, IL-18, IL-1β, Porcine alveolar macrophages.

Introduction

Brucellosis is one of the most prevalent zoonotic infections worldwide. Brucella infections remain intractable in many parts of the world (Ahmed *et al.*, 2016, Baldi *et al.*, 2013, Barquero-Calvo *et al.*, 2015). In recent years, *Brucella suis* (*B. suis*) vaccine strain S2 with reduced virulence was obtained by serial transfer of a virulent *B. suis* biovar 1 strain in China, the mechanisms underlie virulence attenuation of S2 are still unknown (Billard *et al.*, 2007, Cardoso *et al.*, 2006). The rough mutants do not enter host cells by using lipid rafts, contrary to smooth strains. Thus, the *B. suis* LPS might be a major factor that governs the early behavior of bacteria inside macrophages (Cui *et al.*, 2017, Czibener *et al.*, 2016, Di *et al.*, 2016) .

Cysteine-aspartic protease (caspase)-11 and the human orthologues caspase-4/caspase-5 were recently identified as components of the 'non-canonical inflammasome' that senses intracellular LPS derived from Gram-negative bacteria during macrophage-mediated inflammatory responses. *In vivo* studies have clearly shown that caspase-$11^{-/-}$ mice are more resistant to endotoxic septic shock by excessive LPS challenge, which results in pyroptosis and the secretion of interleukin-1β (IL-1β) and IL-18 (Dinarello, 2011). Oxidized phospholipids (oxPAPC) and LPS bind caspase-11 via distinct domains and elicit different inflammasome-dependent activities. Both lipids induce caspase-11-dependent IL-1 release, but only LPS induces dendritic cells (DCs) pyroptosis (Zanoni *et al.*, 2016).

Brucella infection stimulates a robust inflammatory response. In most cases of septic arthritis and osteomyelitis, the inflammatory response elicited by *Brucella* infection induce bone and joint damage (Amit-Kumar *et al.*, 2018, Ke *et al.*, 2015). To elucidate the mechanism of how *B.suis* LPS affects the secretion of proinflammatory factor IL-1, IL-18 and IL-1β from macrophages, we analyzed the expression of caspase-11 in PAM cells stimulated with *B.suis* LPS, the results showed that caspase-11 expression was up-regulated in PAM cells stimulated with *B.suis* LPS. We also demonstrate that specific siRNA targeting caspase-11 effectively inhibits *B.suis* LPS stimulated IL-1, IL-18 and IL-1β release from PAM cells. Our studies provide conclusive

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mechanistic evidence for caspase-11, which plays important roles during innate immune responses of macrophage stimulated with *Brucella* LPS. And our findings might provide important information for the the development of specific anti-inflammatory adjunctive therapies of brucellosis.

MATERIALS AND METHODS

Bacterial strains

The *B. suis* S2 strain purchased from China Institute of Veterinary Drug Control, prepared as previously described (Lei *et al*., 2012).

5% of CO_2 and 85% of relative humidity were used to cell culture

PAM cells were routinely grown at 37°C and 5% CO_2 in DMEM (Gibco BRL, USA) containing 10% heat-inactivated FBS (Gibco BRL, USA) supplemented with penicillin (100 units/mL) (Gibco BRL, USA), and streptomycin (100 µg/mL) (Gibco BRL, USA).

B.suis LPS extraction and cell stimulation

The *B. suis* strains were cross-cryopreserved and cultured in non resistant TSA solid medium, 37°C, after 72 h, single colonies were inoculated in 5 mL resistant TSB liquid medium, then 1:1000 inoculated in 10 mL resistant TSB, when the OD value was about 0.556, An LPS Extraction Kit (iNtRON Biotechnology, Seongnam-Si, Korea) was used to extract *B.suis* LPS according to the manufacturer's instructions. An LPS ELISA Kit (Uscan life science, Houston, USA) was used to determine the concentration of *B.suis* LPS. To check the purity of extracted *B.suis* LPS, SDS-PAGE and silver staining were performed. Briefly, proteinase K (2.5 µg per 1 µg LPS) was mixed with LPS and incubated at 50°C for 30 min and preparations of proteinase K digests were subjected to SDS-PAGE with a 5% (wt/vol) tacking and a 12% (wt/vol) separating gel. Five micrograms LPS from *E. coli* O111:B4 serotypes were obtained from Sigma-Aldrich and used as a control. Following electrophoresis, LPS was visualized by silver staining as described previously (Tsai *et al*., 1982). PAM cells were seeded into 12-well culture plates (8×10^5 cells/well) at 0 h, stimulated by *B.suis* LPS at the concentrations of 0.1, 1, 10, 100, 1000 µg/mL after 24 h, harvested at 48 h. And then, the concentration of 100 ng/mL *B. suis* LPS was used for stimulation of the time points of 3, 6, 12, 24, 48 and 96 h.

siRNAs transfection

PAM cells were seeded into 12-well culture plates (8×10^5 cells/well) at 0 h, to optimize siRNA transfection efficiency, we used X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) to transfect with the FITC-siRNA (Santa Cruz Biotechnology, CA, USA) at 12 h post-seeding, optimizing the ratio of X-tremeGENE siRNA Transfection Reagent and FITC-siRNA obtained a more than 90% transfection efficiency measured by flow cytometry. Caspase-11 siRNA targeting caspase-11 (Santa Cruz Biotechnology) siRNAs or Control siRNA-A (scramble siRNA, Santa Cruz Biotechnology) were introduced into PAM cells as described previously (Jiao *et al*., 2016) and according to the instructions of X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) .

Protein extraction and western blot analysis

The total protein was extracted, western blotting conducted as described previously (Jiao *et al*., 2016). The primary antibodies included rabbit anti-Swine caspase-11 monoclonal antibody (mAB) (1:1000 dilution; Santa Cruz Biotechnology, USA) and rabbit anti-GAPDH mAb (1:5000 dilution; Abcam, USA) as a control. Secondary antibodies were horseradish peroxidase (HRP)-labeled rabbit anti-rabbit IgG H&L (1:5000 dilution; Cell Signaling Technology, USA) and HRP-labeled goat anti-rabbit IgG (1:5000 dilution; Cell Signaling Technology, USA).

Cytokine ELISA assay

In our study, stimulatory concentrations of *B. suis* LPS were 0, 1, 10, 100 and 1000 ng/mL. Based on the *B. suis* LPS dose-dependent IL-1, IL-18 and IL-1β secretion analysis results, *B. suis* LPS time-dependent IL-1, IL-18 and IL-1â secretion analysis was performed, with stimulation times of 0, 12, 24, 36 and 48 h. Swine IL-1, IL-18 and IL-1â ELISA Kits (R&D System, UK) were used to determine the concentrations of IL-1, IL-18 and IL-1β from PAM cells according to the manufacturer instruction using Model 680 Microplate Reader (Bio-Rad, USA).

Cytokine ELISA of *B. suis*-LPS stimulation of PAM cells pretreated with caspase-11 siRNAs

PAM cells were incubated with 100 ng/mL *B. suis* LPS for 24 h (at 24 h post-transfection, LPS was added into the medium) and the supernatant from transfected and non-transfected PAM cells were collected at 48 h post-transfection with Caspase-11 siRNA to determine the inhibitory effects of caspase-11 siRNA on IL-1, IL-18 and IL-1β production. A Swine IL-1, IL-18 and IL-1β ELISA Kit (R&D System, UK) was used to determine the concentrations of IL-1, IL-18 and IL-1β from PAM cells According to the instructions of the manufacturer using Model 680 Microplate Reader (Bio-Rad, USA).

Statistical analysis

The statistical significance of differences between mean values for the various experimental groups and controls was determined as previously described (Jiao *et al*., 2016). Values of $P < 0.05$ were considered significantly different (*) and $P<0.01$ indicated high statistical differences (**).

RESULTS AND DISCUSSION

B. suis LPS-dose dependent expression of caspase-11

Western-blot was used for assessing caspase-11 protein expression in PAM cells stimulated with different concentrations *B. suis*, GAPDH was used as a loading control. *B. suis* LPS dose-dependently increased caspase-11 protein expression, which peaked at 100 ng/mL and

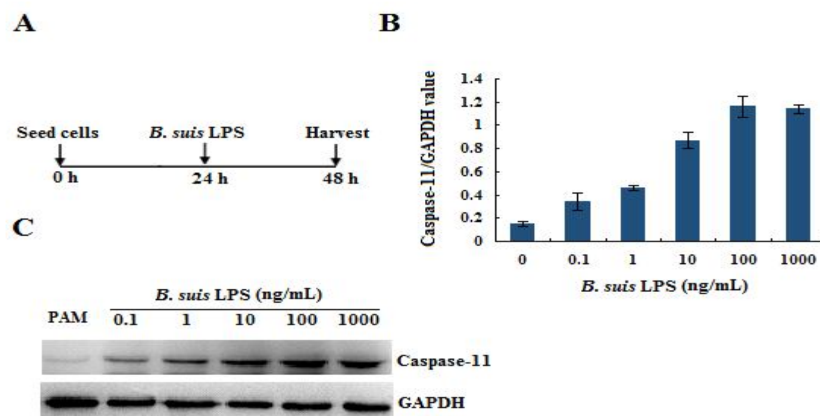

Fig 1: *B. suis* LPS dose-dependent expression of caspase-11 in PAM cells. **(A)** PAM cells were seeded at 0 h and after 24 h, PAM cells were stimulated with increasing concentrations of *B. suis* LPS (0.1, 1, 10, 100 and 1000 ng/mL). At 48 h, the cells were harvested. **(B)** Total protein was extracted from harvested PAM cells and caspase-11 protein expression in PAM cells was determined by western blotting. GAPDH was used as a loading control. **(C)** Quantification of the western blots with B and Scan 5.0 (n=3).

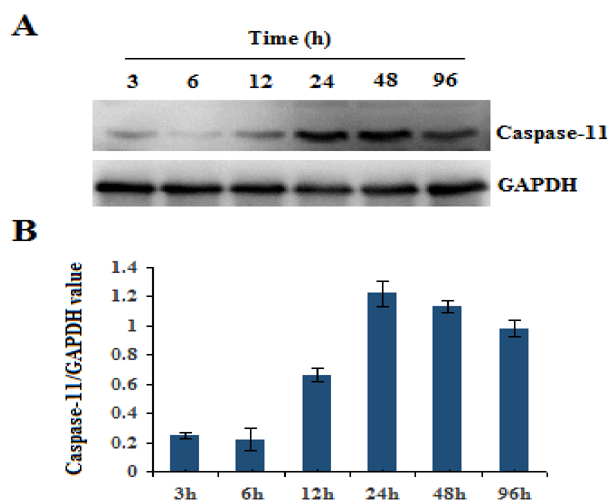

Fig 2: *B.suis* LPS time-dependent expression of caspase-11 in PAM cells. **(A)** PAM cells were seeded at 0 h, and after 24 h, PAM cells were stimulated with *B.suis* LPS (100 ng/mL). And after different stimulation times (3, 6, 12, 24, 48 and 96 h), total protein was extracted from harvested cells and caspase-11 protein expression in PAM cells was determined by western blotting. GAPDH was used as a loading control. **(B)** Quantification of the western blots with B and Scan 5.0 (n=3).

remained stabled at 1000 ng/mL in PAM cells (Fig 1). Then, we assessed caspase-11 protein expression in PAM cells stimulated with *B. suis* LPS for different incubation times (0, 1, 3, 6, 12, 24 and 48 h) and observed that caspase-11 protein expression peaked at 6 h (Fig 2).

Caspase-11 knockdown in *B. suis* LPS-stimulated PAM cells

PAM cells were transfected with caspase-11 siRNA and control siRNA at 12 h, stimulated with LPS at 24 h and harvested after 48 h. As a negative control, the knockdown effects of caspase-11 siRNA on caspase-11 protein expression in PAM cells in the absence *B. suis* LPS stimulation were assessed by western blotting, using equal amounts of protein harvested from PAM cells, PAM cells transfected with scramble siRNA (NC) and PAM cells transfected with caspase-11 siRNA. PAM cells were stimulated with *B. suis* LPS. At 24 h, *B. suis* LPS (100 ng/mL) was added into the medium (stimulation time was 24 h). At 48 h, the PAM cells were harvested. Caspase-11 protein expression in PAM cells transfected with caspase-11 siRNA was significantly reduced compared with PAM and NC groups (Fig 3).

Cytokine ELISA of *B. suis* LPS-stimulated PAM cells

According to the instructions of the manufacturer, Quantikine Swine IL-1, IL-18 and IL-1β Immunoassay Kits. At 24 h, increasing the concentration of *B. suis* LPS incubation increased the IL-1, IL-18 and IL-1β concentration in the supernatants, the supernatants were harvested at 48 h. *B. suis* LPS dose-dependently increased caspase-11 protein expression, which peaked at 100 ng/mL. Therefore, 100 ng/mL was used as the stimulation concentration for IL-1, IL-18 and IL-1β secretion analysis (Fig 4). After stimulation with *B. suis* LPS (100 ng/mL) for different incubation times (0, 12, 24, 36 and 48 h), supernatants were collected and IL-1, IL-18 and IL-1β concentration was determined. At 24 h stimulation, the IL-1, IL-18 and IL-1β secretion effects under *B. suis* LPS stimulation were abrogated. Therefore, 24 h was used as the stimulation time of *B.suis* LPS (100 ng/mL) for further experiments (Fig 5).

Cytokine ELISA of B. suis LPS-stimulated PAM cells pretreated with siRNAs

In our study, we examined whether caspase-11 knockdown affected *B. suis* LPS-mediated cytokine secretion in PAM cells. PAM cells were pretreated with caspase-11 siRNA at 12 h, then the cells were stimulated with *B. suis* LPS at 24 h and the supernatants were harvested at 48 h. The results indicated that the concentrations of IL-1, IL-18 and IL-1β of

caspase-11 siRNA pretreated group were lower than that of control significantly (Fig 6).

Each species of the *Brucella* genus has a particular tropism toward different mammals being the most relevant for human health *B. abortus*, *B. melitensis* and *B. suis* that infect bovines, goats/camelids and swine respectively, *B. suis* induced the production of pro-inflammatory cytokines (Sahoo *et al*., 2011). *Brucella* and this classification is mainly based on the difference in pathogenicity and in host preference, the strains that are pathogenic for humans (*B. abortus*, *B. suis*, *B. melitensis*) carry a smooth LPS involved in the virulence of these bacteria (Porte *et al*., 2003, Losa *et al*., 2018).

Noncanonical inflammasome activation is mediated by caspase 4/11, which recognizes intracellular LPS and promotes pyroptosis and secretion of proinflammatory cytokines. *Brucella* species are infectious intracellular pathogens that replicate in professional and nonprofessional phagocytic cells and subvert immune responses for chronic persistence in the host. Since TcpB suppresses both TLR4 and caspase-11 mediated inflammation, TcpB attenuated LPS induced noncanonical inflammasome activation and suppressed pyroptosis and secretion of IL-1 and IL-1β (Jakka *et al*., 2017).

To elucidate the inflammatory response mechanism of macrophages infected with *B. suis*, *B. suis* LPS was extracted from *B. suis* S2 and caspase-11 was, for the first time, identified as an important functional molecule in *B. suis* LPS-induced IL-1, IL-18 and IL-1β pro-inflammatory cytokine secretion from macrophages.

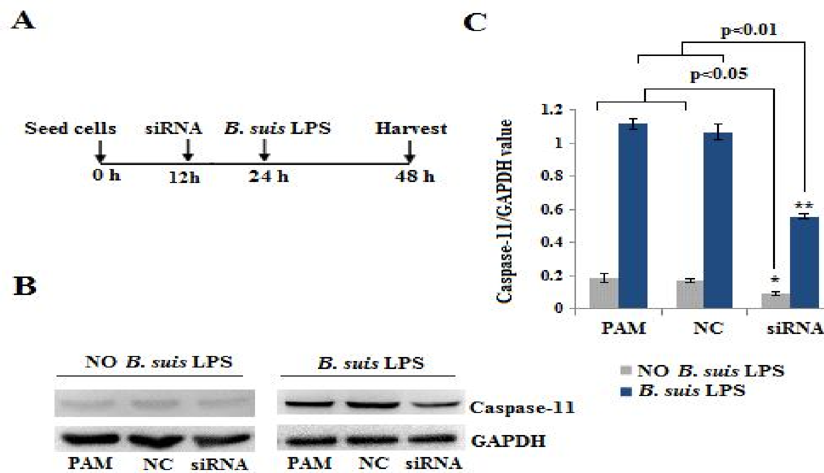

Fig 3: siRNA knockdown reduced in the presence or absence of *B.suis* LPS-induced caspase-11 protein up-regulation in PAM cells. **(A)** PAM cells were seeded at 0 h, transfected with scramble siRNA (NC) and caspase-11 siRNA at 12 h, stimulated with *B.suis* LPS (100 ng/mL) at 24 h and harvested at 48 h. **(B)** The total proteins were extracted from mock or caspase-11 siRNA transfected PAM cells and western blotting was performed to analyze caspase-11 protein expression. GAPDH was used as a loading control. **(C)** Quantification of the western blots with BandScan 5.0 (n=3). P <0.05 were considered significantly different (*) and P<0.01 indicated high statistical differences (**).

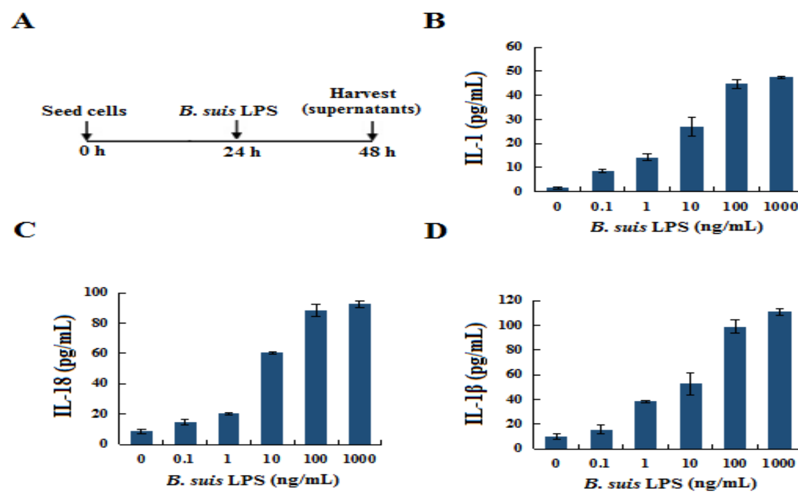

Fig 4: *B.suis* LPS dose-dependent cytokine secretion assays by ELISA. **(A)** PAM cells were seeded at 0 h, stimulated with increasing concentrations of *B.suis* LPS (0, 0.1, 1, 10, 100 and 1000 ng/mL) at 24 h, the supernatants were harvested for cytokine secretion assays at 48 h. **(B)** The analysis of *B.suis* LPS dose-dependent IL-1 (n=3). **(C)** The analysis of *B.suis* LPS dose-dependent IL-18 (n=3). **(D)** The analysis of *B.suis* LPS dose-dependent IL-1β (n=3).

In the early stages of *Brucella* infection, it minimizes the host pro-inflammatory response, and attempt to establish the mechanism of chronic infection, which allows the *Brucella* intracellular survive and replication (Aparajita *et al.*, 2018, Roset *et al.*, 2013). The research identifying the key cellular regulators of cytokine secretion of macrophages under the stimulation of *Brucella* LPS will helpful to the development of novel anti-inflammatory targets and specific anti-inflammatory adjunctive therapies of brucellosis. The results demonstrated that caspase-11 plays an important roles in

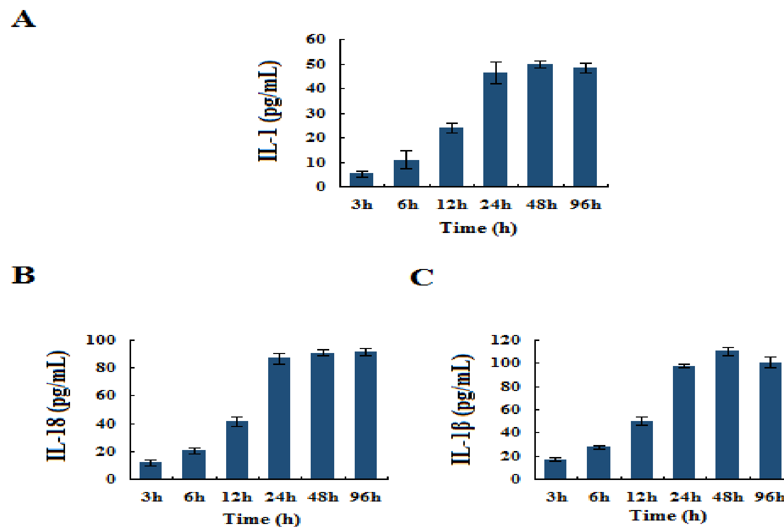

Fig 5: *B.suis* LPS time-dependent cytokine secretion assays by ELISA. PAM cells were seeded at 0 h, stimulated with *B.suis* LPS (100 ng/mL) at increasing time (3, 6, 12, 24, 48 and 96 h), the supernatants were harvested for cytokine secretion assays. **(A)** The analysis of *B.suis* LPS time-dependent IL-1 (n=3). **(B)** The analysis of *B.suis* LPS time-dependent IL-18 (n=3). **(C)** The analy sis of *B.suis* LPS time-dependent IL-1β (n=3).

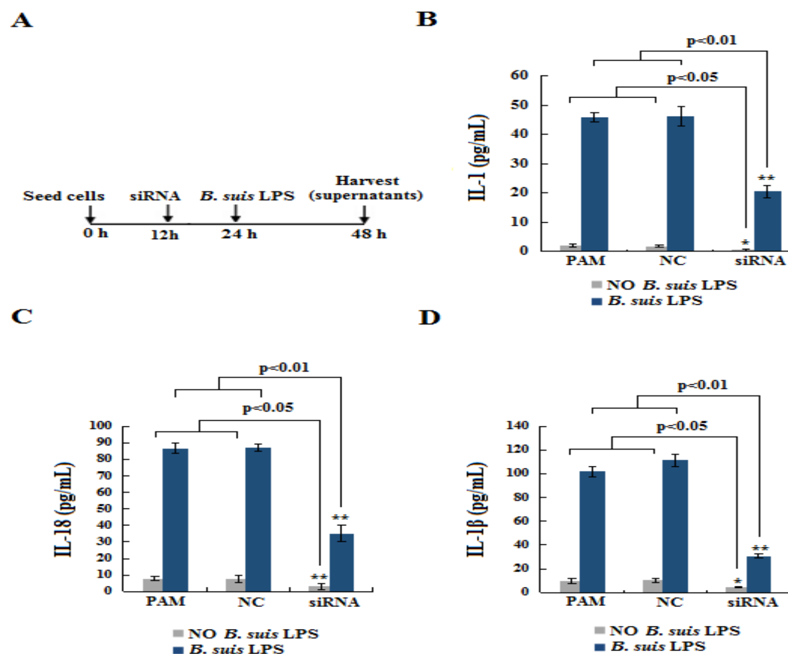

Fig 6: Caspase-11 siRNA reduced *B.suis* LPS induced cytokine secretion. **(A)** PAM cells were seeded at 0 h, transfected with scramble siRNA (NC) and caspase-11 siRNA at 12 h, stimulated *B.suis* LPS (100 ng/mL) at 24 h, the supernatants were harvested for cytokine secretion assays at 48 h. **(B)** Caspase-11 siRNA reduced *B.suis* LPS (100 ng/mL) induced IL-1 secretion (n=3). **(C)** Caspase-11 siRNA reduced *B.suis* LPS (100 ng/mL) induced IL-18 secretion (n=3). **(D)** Caspase-11 siRNA reduced *B.suis* LPS (100 ng/mL) induced IL-1β secretion (n=3). P <0.05 were considered significantly different (*) and P<0.01 indicated high statistical differences (**).

the secretion of pro-inflammatory cytokines of *B. suis* LPS-stimulated porcine alveolar macrophages cells. And our findings might provide an entirely new innate immune response mechanism underlying macrophages dysfunction during *Brucella* infection.

CONCLUSION

In summary, these data indicated that *B. suis* LPS increased IL-1, IL-18 and IL-1β secretion via caspase-11 and these findings might aid our understanding of the interaction between *B. suis* LPS and macrophages.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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