

## RESEARCH ARTICLE

# Deletion of *lpsA* Gene of *Brucella melitensis* Strain M5-90 Promotes Caspase-11 Induced Non-classical Pathways Pyroptosis in *Brucella*-infected Mouse Macrophage Cells

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## Abstract

*Brucella* utilizes diverse virulence factors to modulate the infectious cycle and lifestyle associated with eukaryotic hosts. Lipopolysaccharides (LPS) play an important role in the establishment of persistent infections of *Brucella*, but its mechanism in host cell to interfere with the host's specific signaling pathway related to the elimination of *Brucella* is still not clear. *LpsA*, a glycosyl transferase, involves in the biosynthesis of LPS, and may affect the intracellular survival of *Brucella*. This study aimed to investigate the effects of *lpsA* on pyroptosis in *Brucella*-infected macrophage cells. We constructed the *lpsA* mutant strain (M5-90Δ*lpsA*) and the complementary strain (M5-90Δ*lpsA*-C) of *Brucella melitensis* strain M5-90 infected mouse macrophages to detect pyroptosis. We found that the inactivation of the *lpsA* gene weakened the ability of LPS gains access to cytosol during *Brucella* mutant infections, and reduced the survival of *Brucella*. Western blot and quantitative real-time PCR assays showed that the mRNA and protein levels of caspase-11 and NOD-like receptor family pyrin domain-containing 3 in M5-90Δ*lpsA* group were higher than those observed in M5-90 group. These results indicated M5-90Δ*lpsA* promoted the pyroptosis of RAW264.7 cells (a mouse macrophage cell line) after *Brucella* infection. The finding reveals that *Brucella lpsA* plays a partial role in innate immunity and inflammatory response by inhibiting the LPS-induced atypical pyroptosis pathway.

**Keywords:** *Brucella*, *lpsA*, Survive, Pyroptosis

## *Brucella melitensis* M5-90 Suşunun *lpsA* Geninin Silinmesi *Brucella* İle Enfekte Fare Makrofaj Hücrelerinde Kaspaz-11 ile İndüklenen Klasik Olmayan Piroptozis Yolaklarını Teşvik Eder

## Öz

*Brucella*, ökaryotik konakçılarla ilişkili enfeksiyon döngüsünü ve yaşam biçimini düzenlemek için çeşitli virülens faktörlerini kullanır. Lipopolisakaritler (LPS), *Brucella*'nın kalıcı enfeksiyonlarının oluşmasında önemli bir rol oynar, ancak konakçı hücrede, *Brucella*'nın ortadan kaldırılmasıyla ilgili konakçının spesifik sinyalizasyonuna müdahale etme mekanizması halen net değildir. Bir glikozil transferaz olan *LpsA*, LPS'nin biyosentezinde görev alır ve *Brucella*'nın hücre içi yaşama yeteneğini etkileyebilir. Bu çalışmada *lpsA*'nın *Brucella* ile enfekte makrofaj hücrelerinde piroptoz üzerine etkilerinin araştırılması amaçlanmıştır. Piroptozu saptamak için *Brucella melitensis* M5-90 suşunun *lpsA* mutanı (M5-90Δ*lpsA*) ve komplementer suşu (M5-90Δ*lpsA*-C) ile enfekte olmuş fare makrofajları oluşturduk. *LpsA* geninin inaktivasyonunun, LPS'nin *Brucella* mutant enfeksiyonları sırasında sitozole giriş yeteneğini zayıflattığını ve *Brucella*'nın yaşama yeteneğini azalttığını saptadık. Western Blot ve kantitatif real-time PCR deneyleri, M5-90 grubuna oranla M5-90Δ*lpsA* grubunda kaspaz-11 ve NOD-benzeri reseptör ailesi pyrin domain-containing 3'ün mRNA ve protein seviyelerinin daha yüksek olduğunu gösterdi. Bu bulgular, M5-90Δ*lpsA*'nın, *Brucella* enfeksiyonu sonrası bir fare makrofaj hücre hattı olan RAW264.7 hücrelerinde piroptozu yol açtığını gösterdi. Bu bulgu, *Brucella lpsA*'sının, LPS ile indüklenen atipik piroptoz yolunu inhibe ederek doğal bağışıklık ve enflamatuvar yanıtta kısmi bir rol oynadığını ortaya koymaktadır.

**Anahtar sözcükler:** *Brucella*, *lpsA*, Canlılık, Piroptozis

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## INTRODUCTION

Brucellosis is an important zoonotic disease caused by *Brucella*, a Gram-negative facultative intracellular bacterium, listed as a Class B bioterrorism agent by the U.S. Centers for Disease Control and Prevention (CDC) [1]. Virulence factors are the key for *Brucella* to survive and reproduce in host cells successfully [2], a variety of strategies can be used to establish and maintain the long-term survival in host cells for *Brucella*. They can escape the inflammatory response in the early stages of infection [3,4], once enter the host cell, they affect intracellular transport and activation, and utilize some of the environmental pressures encounter (such as acidic environments and nutritional stress) as a stimulus for gene induction to change them intracellular transport [5]. Eventually, *Brucella* deprives the ability of cells for processing antigens and resisting apoptosis to benefit its survival [6,7].

Lipopolysaccharide (LPS) is one of the important virulence factors for the survival of *Brucella*, maintains the structural and functional integrity of bacterial outer membrane proteins, but also plays a role in the immune escape, it is crucial for the integrity of bacterial virulent [8]. When O-polysaccharide of *Brucella* LPS is inactivated, it changes from smooth type to rough type, resulting in weakened bacterial virulence and easy to be killed by macrophages [9,10]. LPS-based O-type polysaccharides inhibit cell phagocytosis, lysosomal lysis and host cell apoptosis [11]. The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome belongs to the NOD-like receptor (NLR) family and composed of NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and pre-caspase-1 [12]. NLRs or the absent in melanoma 2 (AIM2) protein receive danger signals from extracellular or intracellular, leading to the assembly and activation of inflammasomes, which in turn activate caspase-1 [13]. Activated caspase-1 causes cytokines such as interleukin (IL)-1 $\beta$  and IL-18 to mature and activate [14]. However, recent researches have shown that Gram-negative bacteria can trigger the NLRP3 inflammatory response in a nonclassical way that relies on caspase-11 belongs to the family of inflammatory caspases [15,16]. The caspase recruitment domain (CARD) of this type of caspase can directly recognize and bind to LPS, and undergo self-oligomerization to show obvious protease activity [17]. Caspase-11 activated by LPS cleaves gasdermin-D (GSDMD), in the junction between the N-terminal and C-terminal domains, thereby releasing the GSDMD N-terminal domain (GSDMD-N) with pore-forming activity [18]. GSDMD-N is transferred to the mass by binding membrane phosphoinositide membrane, perforation in the membrane causes the cell membrane to rupture and cell death termed pyroptosis [19]. The recent research demonstrates that *B. abortus* can trigger the activation of non-conicaase-dependent caspase-11 and GSDMD and *Brucella*-LPS is the ligand for caspase-11 activation. It is determined that

*B. abortus* can trigger the formation of cell pyroptosis [20]. Unlike the other genes related to LPS synthesis, *lpsA* is a glycosyl transferase, involved in the biosynthesis of the inner core encoding LPS, which may affect the intracellular survival of *Brucella*, but the specific function is unknown [21]. Studies have shown that stimulated with LPS, macrophages are activated in a proinflammatory direction via the activation of inflammatory cysteinyl aspartate specific proteinase, promoting the release of cellular inflammatory factors and cause cell pyroptosis [22]. However, the mechanism by which *Brucella* LPS regulates the occurrence of pyroptosis in host cells is still unknown.

Our study aims to research the function of *lpsA* in pyroptosis during *B. melitensis* strain M5-90 infection in host cells. In the present study, the roles of *lpsA* in pyroptosis were detected. Besides, after RAW264.7 cells were infected with M5-90 $\Delta$ *lpsA* mutant, the activation of inflammatory and secretion of the inflammatory cytokines IL-18 and IL-1 $\beta$  was examined.

## MATERIAL AND METHODS

### Bacterial Strains, Plasmids and Cell Line

*B. melitensis* strain M5-90 was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). All *Brucella* strains were cultured in *Brucella* Agar or *Brucella* Broth (Difco, MI, USA) at 37°C in 5% CO<sub>2</sub>. *Escherichia coli* strains DH5 $\alpha$  and BL21 were grown on Luria-Bertani (LB) medium (Madison, WI, USA) at 37°C. If necessary, the culture media were supplemented with appropriate kanamycin and ampicillin (100  $\mu$ g/mL) for the mutant. pBBR1MCS4 vector was purchased from Promega (Madison, WI, USA). Murine (or mouse) macrophage RAW264.7 cells (Lu Ao, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) supplemented with 10% fetal bovine serum (Taixin, Beijing, China) at 37°C with 5% CO<sub>2</sub>. All *Brucella* strains were manipulated in a biosafety level 3 laboratory.

### Construction of M5-90 $\Delta$ *lpsA* mutant

The M5-90 $\Delta$ *lpsA* deletion mutant was constructed and modified according to the methods previously reported [23] and primers were shown in Table 1. We used homologous recombination to construct mutant strains, briefly. Design of primers for upstream and downstream homology arms was achieved with software Primer 6.0 (the length of the homology arm sequence is 350-550 bp). After the primer design is completed, the reverse complement sequence of the first 19 bases of the kanamycin resistance gene sequence was added to the 5' end of the downstream primer of the upstream homology arm and the kanamycin resistance gene was artificially added to the 5' end of the upstream primer of the downstream homology arm the last 20 bases of the sequence. Blunt-end enzyme PCR was

Table 1. Primers of mutants used for this work	
IpsA-N-F	AGGATACCGGCGTCGGCATT
IpsA-N-R	GACATTCATCCCAGGTGGCTAATTCGCTTGCCCTCA
IpsA-C-F	TCTGGGGTTCGAAATGACCGAGCGGTTCCACTTTTACACAG
IpsA-C-R	CTGACCCGCGCGCG
kan-F	GCCACCTGGGATGAATGTC
kan-R	CGGTCATTCGAACCCAGAGA

used to amplify the upstream and downstream homology arms of the target gene and the kanamycin resistance gene, and overlap extension PCR was used to fuse the fragments. The mutation box was subcloned into the pMD19-T vector for sequencing. Competent M5-90 was electroporated with the plasmid pMD19-T-IpsA. Mutants were selected in the presence of 100 µg/mL ampicillin and 100 µg/mL kanamycin for the screening. The deletion mutant was confirmed by qRT-PCR amplification.

### M5-90ΔIpsA-C Complementary Strain

The complementary strain M5-90ΔIpsA-C was constructed and modified according to the methods previously described [24]. Genomic DNA from M5-90 was amplified using primers IpsA-A-F (5'-AAGCTTATGATATTGCCCGTATT-3') and IpsA-A-R (5'-GAATTCGCGACACTACTTCTAA-3'). PCR products were cloned into pMD19-T simple vector for sequencing and then subcloned into pBBR1MCS4 to generate the plasmid pBBR4-IpsA. Subsequently, the plasmid pBBR4-IpsA was electroporated into M5-90ΔIpsA, obtaining the complementary strain. The complementary strain was referred to as M5-90ΔIpsA-C.

### Growth Curve of M5-90ΔIpsA Mutant

In order to monitor and analyze the growth of strains in real-time, the M5-90, M5-90ΔIpsA, M5-90ΔIpsA-C monoclonal colonies were picked out respectively and placed them in to *Brucella* broth when OD<sub>600</sub>=0.8. Using sterilized *Brucella* broth the density of them were adjusted to OD<sub>600</sub>=0.1 and cultured in a rotary shaker (250 rpm) at 37°C. The bacterial population was inactivated with adding 4-6% formaldehyde and OD<sub>600</sub> value was measured until each strain culture creates a plateau, and the growth curve was drawn.

### Analysis of Stress Tolerance of M5-90ΔIpsA mutant

In order to assess the adhesion and invasive ability of the mutant, the IpsA mutant was added to infected cells at a multiplicity of infection (MOI) of 100:1, and incubated at 37°C and 5% CO<sub>2</sub> for 15, 30, 45 or 60 min. Next, the cells were washed with phosphate-buffered saline (PBS) to wash away non-adherent bacteria and then incubated in a medium containing 50 units (2.5 µL) of gentamicin for 60 min to kill extracellular *Brucella*. The cells were rinsed with PBS, lysed, and incubated at 37°C for 10 min to release intracellular bacteria. Appropriate concentrations were

prepared by dilution and the ability to adhere and invade macrophages was counted by CFU count. To determine the environmental stress tolerance of the mutant strain, the IpsA mutant strain was cultured to logarithmic growth phase, centrifuged at 12,000 rpm for 10 min and rinsed 2 times with PBS. The bacterium was cultured in liquid medium (1 mL TSB) at 37°C under different stress conditions (in the presences of 1.5 M NaCl and 10 mM H<sub>2</sub>O<sub>2</sub> and at pH 2.5, pH 11.5) for 30 min and at 50°C for 30 min, respectively. The CFUs were then counted and the percentage of surviving bacteria calculated relative to the control and wild-type (WT) (% survival). Under the stress conditions, normally cultured bacteria were used as controls.

### RAW264.7 Cells Infection Assay

RAW264.7 cells were cultured in 6-well plates to form a monolayer with 80% coverage (2.5 x 10<sup>6</sup>). The cells were infected with M5-90, M5-90ΔIpsA, M5-90ΔIpsA-C at 100:1 of MOI and incubated at 37°C and 5% CO<sub>2</sub> for 0 h, 4 h, 12 h and 24 h of infection. The cells were lysed with 0.1% Triton X-100 (Sigma-T8787, USA) at 37°C for 10 min to release intracellular bacteria. The lysate was diluted 10-fold to an appropriate concentration and enumerated the CFU by plating on *Brucella* broth plates.

### Assessing the Ability of LPS Gains Access to the Cytosol During *Brucella* Mutant Infections

To assess the influence of the inactivation of IpsA on the entering process of *Brucella* to the host cell and the cytoplasmic localization of LPS, digitonin was used to isolate the parent strain or mutant strain from the infected cytoplasmic components of RAW264.7 cells with different adhesion periods. The Limulus reagent quantitative method was used for detection of the LPS of *Brucella* into host cells. For lyses, the cells were washed with 1 mL Triton X-100 (0.2% v/v, 100 mL PBS, 200 µL Triton X-100, Sigma-T8787, USA) and incubated at 37°C for 10 min to release the intracellular bacteria. The lysate was diluted 10 times to obtain an appropriate concentration for plate counting. On the other hand, we use a 2-micron filter to filter the cell components, use the fluorescent probe SYTO9 to label the live bacteria, and use confocal laser scanning microscopy (CLSM) to observe.

### Quantitative Real-time PCR (qRT-PCR)

RAW264.7 cells were infected with M5-90, M5-90ΔIpsA or M5-90ΔIpsA-C according to the above-mentioned method. The cells not infected with *Brucella* were used as controls. Extraction of the total RNA from cells infected with *Brucella* mutants at 4 h, 12 h and 24 h was carried out. The RNA samples were treated with DNase I (Thermo Fisher Scientific, Shanghai, China) to remove contaminant genomic DNA. The RNA molecules were subsequently reverse transcribed into cDNA by using PrimeScript<sup>TM</sup>RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, USA). Pyroptosis-associated genes (caspase and NLRP3) were selected for

**Table 2.** Primers of qRT-PCR used for this work

β-actin-F	GAGACCTCAACACCCAG
β-actin-R	GAGCATAGCCCTCGTAGAT
Caspase-11-F	ACAAACACCCCTGACAAACCAC
Caspase-11-R	CACTGCGTTCAGCATTGTAAA
Caspase-1-F	TGCCGTGGAGAGAAACAA
Caspase-1-R	ATGAAAAGTGAGCCCCTG
NLRP3-F	TTGGAGACACAGGACTCAGG
NLRP3-R	CATTGTTGCCAGGTTTCAG

qRT-PCR amplification. The primers used in this scope were listed in Table 2. The qRT-PCR conditions were consisted of 5 min at 95°C for pre-incubation, followed by 40 cycles at amplification (95°C for 30 s, 62°C for 30 s and 72°C for 30 s). The qRT-PCR was carried out with a QuantStudio™ 7 Flex system (Thermo Fisher Scientific, USA). The qRT-PCR reaction volume for per sample was adjusted as 20 μL containing 10 μL 2 × SYBR Premix Ex Taq II (Takara, USA), 0.4 μL of each primer (10 nM), 2 μL cDNA target (10 μg) and 7.2 μL ddH<sub>2</sub>O. All assays were performed three times.

### Western Blotting

The cells infected with *Brucella* were collected and the cell culture supernatant was discarded. The cells were washed with PBS for 3 times and 100 μL non-denatured cell/tissue lysate (PMSF) was added per well. The cells were put on ice and lysed for 10 min. The cells were scraped and sucked into a new microcentrifuge tube. The repeated three wells were combined into one tube and centrifugated at 12,000 rpm for 5 min at 4°C. Protein concentrations were determined using the micro-BCA protein assay (Thermo Fisher Scientific, Shanghai, China). Equal amounts of protein (20 μg) were then resolved on a 12% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (GE Biosciences) for 60 min at 300 mA and blocked in TBS with 0.1% Tween 20 for 1 h at 37°C. The membranes were then probed with primary antibodies (rabbit anti-NLRP3 monoclonal antibodies and anti-caspase-11 polyclonal antibodies) in TBS with 0.05% Tween 20 overnight. After the membrane was washed 3 times in TBS with 0.1% Tween 20 for 7 min, the membranes were incubated with an anti-rabbit IgG antibody conjugated with peroxidase at a dilution of 1: 1000 at 37°C for 3 h in a shaker-incubator. The protein bands were monitored with using protein imaging system (FluorChemE, USA).

### Cytokine Detection

The culture supernatants were collected at different time periods from the RAW264.7 cells infected with M5-90, M5-90Δ*IpsA*, non-infected control and the cells with M5-90Δ*IpsA*-C strain. IL-18 and IL-1β were determined using a Mouse Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All assays were performed in triplicate.

### Statistical Analysis

The data were presented as mean ± SEM (SD). GraphPad Prism software was used to analysis. Data of multiple groups were analyzed using one-way ANOVA followed by Bonferroni post hoc test, while comparisons between two groups were performed by unpaired Student's *t*-test. Statistical significance was defined as *P*<0.05.

## RESULTS

### Construction of M5-90Δ*IpsA* Mutant and M5-90Δ*IpsA*-C Complementary Strain

The M5-90Δ*IpsA* mutant was constructed by homologous recombination and resistance gene replacement, and the complementary strain was successfully confirmed by PCR and resistance screening (Fig. 1-a).

### LPS gains Access to the Cytosol During *Brucella* Infections

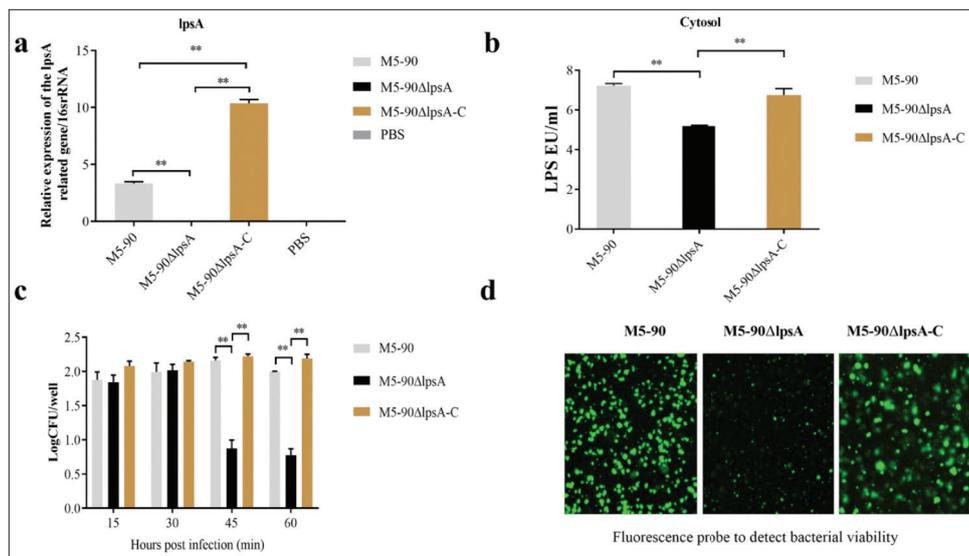
The entry of LPS into the cytosol is a critical step for Gram-negative bacteria to trigger an inflammatory response. We tested whether the deletion of *IpsA* would affect the entry of *Brucella* LPS into the cytosol during infection. We extracted cytosol from uninfected or M5-90, M5-90Δ*IpsA*-infected RAW264.7 cells using digitonin and assessed LPS levels with the Limulus amoebocyte lysate (LAL) assay. The results showed that M5-90Δ*IpsA*-infected macrophages showed a lower level of LPS content than the parental strain (Fig. 1-b) (*P*<0.01). Similarly, the number of bacteria entering the host cell in the initial stage is also reduced (Fig. 1-c,d) (*P*<0.01). The deletion of *IpsA* leads to a decrease in the synthesis of LPS in *Brucella* during infection or few LPS entering the cytoplasm.

### Growth Curve of M5-90Δ*IpsA* Mutant

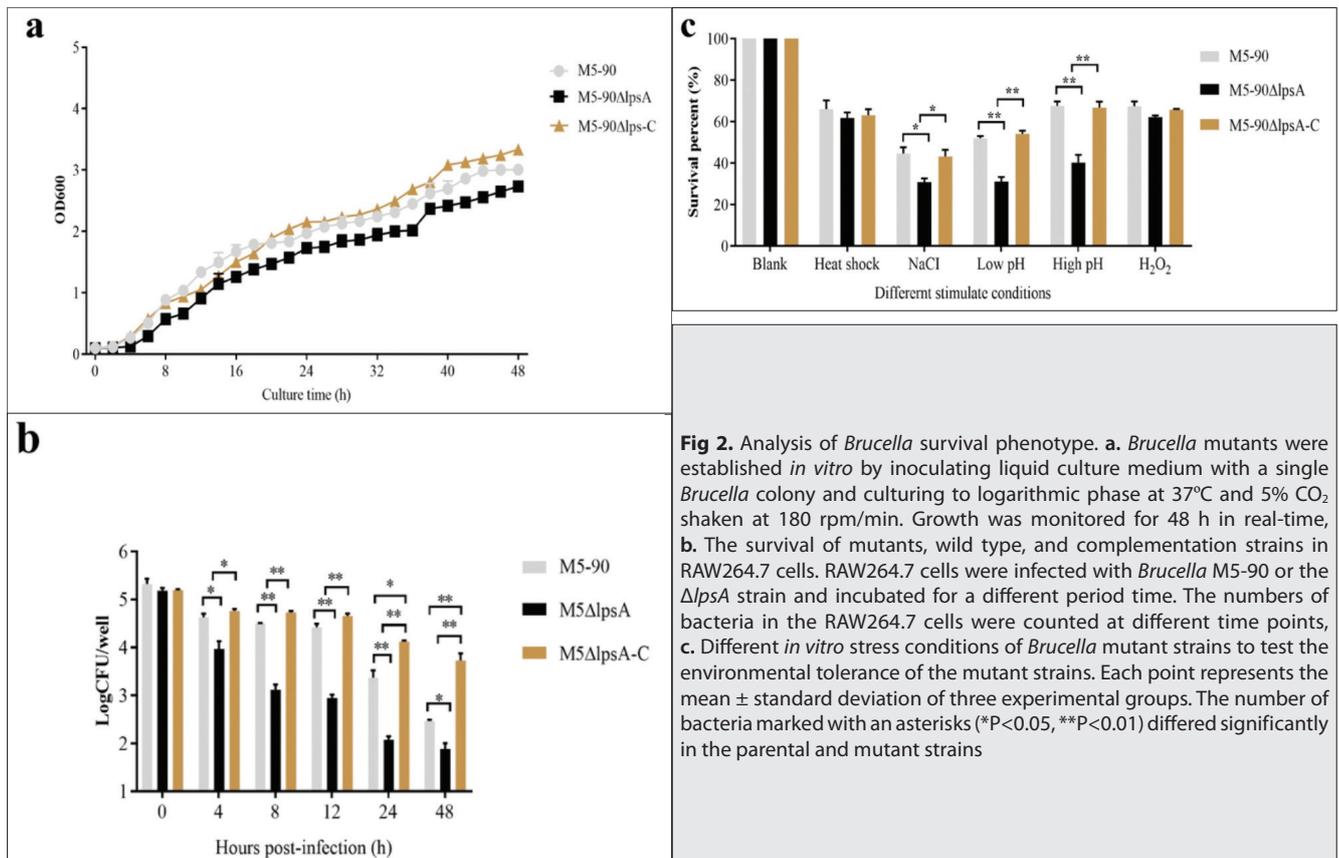
We determined the effect of the absence of *IpsA* on the growth of M5-90 in a liquid medium. Compared with the wild strain, the growth phenotype trend of M5-90Δ*IpsA* mutant is basically the same. All strains have reached the logarithmic growth phase at 16 h, and reach the bacterial growth plateau at 32 h, but the mutant strain grew slowly (Fig. 2-a). Supplementation changed this phenotypic change. These results indicate that *IpsA* affects the growth of M5-90 *in vitro* liquid medium, but the M5-90Δ*IpsA* strain does not lose its basic characteristics.

### Reduced Environmental Tolerance of M5-90Δ*IpsA* Mutant

Since *Brucella* generally survives in chronically hungry and hypoxic niches, we initially studied the physiology of *Brucella* under microaerobic conditions. Under consistent nutritional conditions, we varied the pH, temperature and oxidizing environment conditions of the growth environment, 48 h after inoculation and used the plate



**Fig 1.** LPS gains access to the cytosol during M5-90 infections. **a.** Real-time fluorescent quantitative PCR to detect the transcription level of *lpsA* gene in M5-90, M5-90Δ*lpsA* and M5-90Δ*lpsA*-C, **b.** Extracted cytosol from uninfected or *Brucella*-infected mouse macrophage using digitonin and assessed LPS levels with the LAL assay, **c.** RAW264.7 cells were infected with *Brucella* wild type and mutant strains at different time points and CFUs counted to determine *Brucella* adhesion and invasion, **d.** SYTO9 green fluorescent label live bacteria, CLSM observes the ability of *lpsA* mutant strain to adhere and invade cells. Asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ ) indicate significant differences between the parental and mutant strains

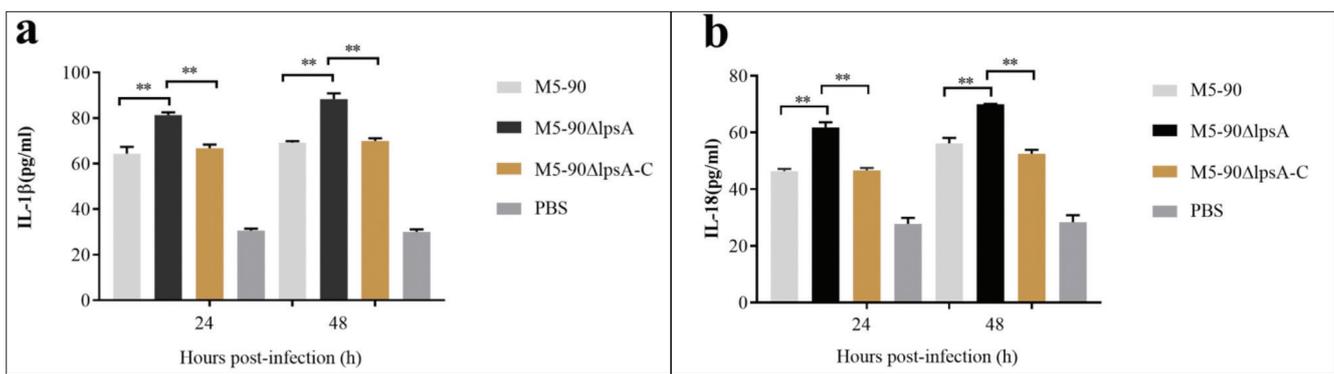


**Fig 2.** Analysis of *Brucella* survival phenotype. **a.** *Brucella* mutants were established *in vitro* by inoculating liquid culture medium with a single *Brucella* colony and culturing to logarithmic phase at 37°C and 5% CO<sub>2</sub> shaken at 180 rpm/min. Growth was monitored for 48 h in real-time, **b.** The survival of mutants, wild type, and complementation strains in RAW264.7 cells. RAW264.7 cells were infected with *Brucella* M5-90 or the Δ*lpsA* strain and incubated for a different period time. The numbers of bacteria in the RAW264.7 cells were counted at different time points, **c.** Different *in vitro* stress conditions of *Brucella* mutant strains to test the environmental tolerance of the mutant strains. Each point represents the mean ± standard deviation of three experimental groups. The number of bacteria marked with an asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ ) differed significantly in the parental and mutant strains

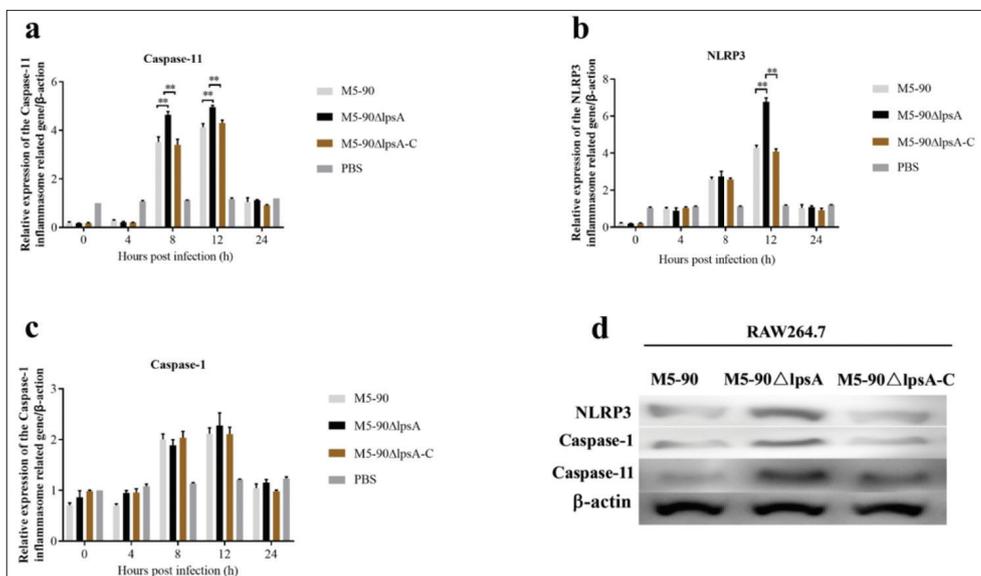
counting method to compare bacterial growth. The results showed that when compared with the parent strain the LPS mutant strain is more sensitive to environmental osmotic pressure (Fig. 2-c) ( $P < 0.05$ ) and pH (Fig. 2-c) ( $P < 0.01$ ).

### M5-90Δ*lpsA* Mutant is Attenuated in RAW 264.7 Cell

In order to evaluate the survival of the mutant strain in the cell after the deletion of *lpsA*, RAW264.7 cells were infected



**Fig 3.** Levels of IL-18 and IL-1 $\beta$  released from RAW264.7 cells infected by M5-90 $\Delta$ *IpsA*. After *Brucella* mutants infect cells, the release of IL-18 and IL-1 $\beta$  was measured at 24 and 48 h with ELISA. Each point represented the mean  $\pm$  standard deviation of three experimental groups. Asterisk (\* $P$ <0.05, \*\* $P$ <0.01) indicates significant differences in the release of IL-18 and IL-1 $\beta$  between the parental and mutant strains after infection



**Fig 4.** Transcription and expression of NLRP3 and caspase-1/11 in RAW264.7 cells infected by M5-90 $\Delta$ *IpsA*. **a**, **b**, **c**. qRT-PCR detection of *Brucella* infected cells indicate the relative expression levels of inflammasome NLRP3 and caspase-1/11 genes. **d**. Western blot analysis of cell protein lysates from RAW264.7 cells infected with M5-90 $\Delta$ *IpsA* and M5-90 $\Delta$ *IpsA-C* using anti-NLRP3, caspase-1/11 primary antibodies to determine NLRP3 and caspase-1/11 protein levels, with  $\beta$ -actin as a loading control. Samples were from the same experiment and blots were processed in parallel. Each point represented the mean  $\pm$  standard deviation of three experimental groups. Asterisk (\* $P$ <0.05, \*\* $P$ <0.01) indicates significant differences in the mRNA level of NLRP3 and caspase-1/11 between the parental and mutant strains after infection

with M5-90 $\Delta$ *IpsA* and the wild type strains at an MOI of 100, to assess the survival and replication ability of the mutant strain in macrophages. There was no difference in the number of surviving bacteria in RAW264.7 cells after 0 h of infection, but after 4 h of infection, when compared with M5-90, the number of M5-90 $\Delta$ *IpsA* bacteria in macrophages decreased by 1.0 log CFU ( $P$ <0.05). After 48 h of infection, this difference increased up to 2.5 log CFU ( $P$ <0.05). The above results indicate that compared with the wild strain (Fig. 2-b), the mutant strain is attenuated in macrophages and reduced the RAW264.7 cells. The ability to replicate in cells indicates that *IpsA* is involved in the chronic infection of *Brucella*.

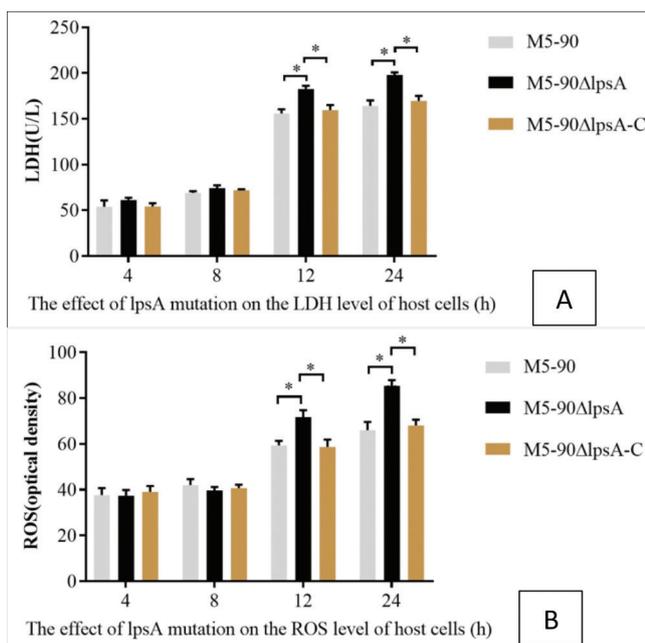
#### Pro-inflammatory Cytokine Expression in RAW264.7 Cells

#### was Increased in the Absence of *IpsA*

We determined whether M5-90 $\Delta$ *IpsA* could affect IL-1 $\beta$ , IL-18 in RAW264.7 cells. The results show that the levels of cytokines IL-1 $\beta$ , IL-18 were higher in M5-90 $\Delta$ *IpsA* infected RAW 264.7 cells than in M5-90 infected cells ( $P$ <0.01) (Fig. 3-a,b).

#### M5-90 $\Delta$ *IpsA* Promotes Caspase-11 Induces Non-classical Pathways Pyroptosis

We detected the transcriptional expression of caspase-1/11 and NLRP3 in M5-90, M5-90 $\Delta$ *IpsA* and M5-90 $\Delta$ *IpsA-C* at the mRNA level and protein level, respectively. 12 h after the infection, the relative expression levels of caspase-11 and NLRP3 mRNA in cells infected with M5-90 $\Delta$ *IpsA* were



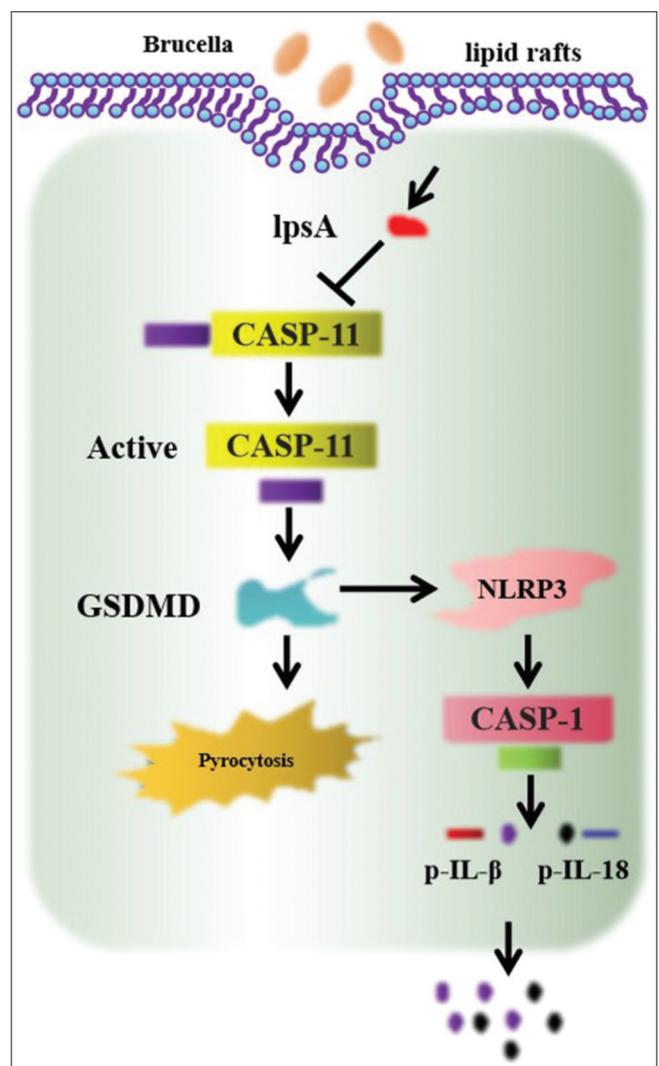
**Fig 5.** Levels of LDH and ROS released from cells infected with *Brucella* M5-90 or *Brucella* M5-90ΔlpsA. LDH (A) and ROS (B) releases were measured at 4, 8, 12 and 24 h after *Brucella* infection. Asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ ) indicate differences in LDH and ROS releases between the parental and mutant strains

significantly higher than those in cells infected with M5-90 (Fig. 4-a,b) ( $P < 0.01$ ). At the same time, the expression of caspase-11 protein and NLRP3 was higher than that of M5-90 infection group (Fig. 4-d), while the expression of NLRP3 is enhanced, the inactivation of *lpsA* leads to an increase in reactive oxygen species (ROS) and an increase in lactate dehydrogenase (LDH) levels (Fig. 5-a,b) ( $P < 0.05$ ). However, the expression of caspase-1 is low, indicating that *Brucella* LPS may not be effectively activated (Fig. 4-c,d). In addition, the complementary M5-90ΔlpsA mutant restored these changes. These results indicate that the M5-90ΔlpsA mutant inhibits pyroptosis of RAW264.7 cells by activating the non-classical pathway of caspase-11.

Therefore, our results preliminarily prove that in the intracellular life cycle of *Brucella*, the *Brucella lpsA* gene, as a key virulence gene, can increase *Brucella* viability by inhibiting the LPS-induced atypical pyroptosis pathway in the host cell. Different from the classic caspase-1 activation of cell pyroptosis pathway, LPS can activate caspase-11 by directly binding to the conserved structure of LPS lipid A. The activated caspase-11 further cleaves the GSDMD protein and promotes the occurrence of pyroptosis and promotes inflammation. The activation of body NLRP3 and the production of cytokines (Fig. 6). Therefore, *lpsA* plays an important role in the intracellular life cycle of *Brucella*.

## DISCUSSION

LPS is one of the main components of the cell membrane of Gram-negative bacteria and the main component of



**Fig 6.** The model of the process of *Brucella lpsA* regulating the host cell pyroptosis. In the first few hours of infecting macrophages, 90% of *Brucella* are killed by macrophages, and the remaining 10% of *Brucella* form replication vesicles to survive. *Brucella* enters the host and interacts with the giant lipid rafts on the cell membrane of phages interact, *Brucella* and lipid rafts are closely related. After being stimulated by LPS in the cytoplasm, mouse caspase-11 and its human counterpart caspase-4/5 can interact with LPS. The conserved structure of lipid A is activated by direct binding. The activated caspase-11 further cleaves the GSDMD protein and promotes the occurrence of pyroptosis, releasing a large number of cytokines. Our results indicate that the lack of *lpsA* promotes the cell pyroptosis happened

its endotoxin and crucial to the recognition of bacteria by immune cells [25]. However, *Brucella* develops some strategies to evade recognition by the immune system, thereby establishing an infection inside the host. One of these strategies is to modify its LPS to avoid effective recognition by pattern recognition receptors [26]. Due to the atypical structure [27], *Brucella* LPS becomes a special virulence factor. In the invasion of host cells and early survival of *Brucella*, S-type LPS and its O side chain play an important role [28]. After *Brucella* invades the host, *Brucella* forms vesicles in the pre-acidic environment, called *Brucella*-containing vacuole has become a relatively safe living

environment for *Brucella*. Therefore, although *Brucella* uses virulence factors to evade part of the immune recognition of host cells, caspase-11 becomes the second obstacle to LPS recognition to promote cytoplasmic monitoring [29]. Once activated, caspase-11 leads to cell apoptosis and activation of NLRP3 inflammasomes, which in turn leads to the activation of caspase-1 and the release of pro-inflammatory cytokines, which are essential for innate immunity against Gram-negative bacteria [20]. In this study, we proved that *IpsA*, a gene related to the synthesis of lipooligosaccharide, is involved in the process of *Brucella*-induced macrophage pyroptosis.

We analyzed the M5-90Δ*IpsA* mutant, replenishment strain and parental strain, and confirmed that the reduced viability of the mutant is directly related to the *IpsA* gene deletion. We found that the survival rate of M5-90Δ*IpsA* in RAW264.7 cells was impaired. This study showed that the virulence of the *IpsA* mutant was significantly reduced. Our results further support the work of other groups, the *IpsA* gene is involved in the biosynthesis of the core of *Brucella* LPS, and the deletion mutation will cause *Brucella* to transform into an attenuated rough type, *IpsA* mutant was attenuated and was eliminated early in the infection process [21,30].

In the processing of pathogen-host cell interaction, some of the pathogens can interfere with the programmed death of host cells, which is conducive to the microorganism to survival and reproduction [10]. One Study [31] has found that the pathogenic bacterial LPS enters the cytoplasm and the mouse caspase-11 and its human counterpart caspase-4/5 can be activated by direct binding to the conserved structure of LPS lipid A. The activated caspase-4/5/11 further cleavage the GSDMD protein and promote the occurrence of cell pyroptosis [32]. However, the mechanism of the *Brucella* LPS regulating the host cell pyroptosis is still unclear. In this study, M5-90Δ*IpsA*, M5-90Δ*IpsA*-C and M5-90 were used to infect RAW264.7 cells respectively. We used Limulus reagent to detect the LPS content in the infected cells. Compared with the parent strain, cells infected with the mutant strain showed lower LPS content, indicating that *IpsA* may be involved in the important process of *Brucella* transporting LPS into host cells, but the specific mechanism needs to be further explored. In the past few years, great progress has been made in understanding the mechanism of programmed death. Previous reports confirmed that smooth *Brucella* can inhibit macrophage death, while rough *Brucella* can induce apoptosis by activating caspase-2 [33,34]. In contrast, another study observed that smooth *Brucella* induces apoptosis of RAW264.7 macrophages by producing ROS [35]. In this research we confirmed cell death using LDH release assay suggesting that *IpsA* mutant triggers caspase-11-dependent differential pyroptosis compared with parent strain. And we further verified the difference in NLRP3 expression at the mRNA and protein levels, and it is correlated with ROS.

IL-1β as a powerful cell inflammatory factor with a wide range of biological effects in immune response, can promote the proliferation and differentiation of immune cells and can be released to the outside of the cell [36]. IL-18 mediates the cascade of inflammation, initiates the host immune response, and plays a key role in driving inflammation [37]. There will be a large amount of IL-1β and IL-18 produced and released along with the process of pyroptosis. The level of inflammatory cytokine production in M5-90Δ*IpsA* infected cells was significantly higher than that of M5-90 infected cells. Our results further indicate that *IpsA* may inhibit host cell pyroptosis and increase the viability of *Brucella*.

In conclusion, the results showed that i) the IL-18 and IL-1β levels of M5-90Δ*IpsA* were higher than those of M5-90, and ii) the expression level of caspase-11 and NLRP3 in M5-90Δ*IpsA* group was higher than that of M5-90. We preliminarily proved that M5-90Δ*IpsA* can activate caspase-11-mediated cell pyroptosis and promote the occurrence of inflammation.

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#### CONFLICT OF INTEREST

The authors have declared that no competition interests.

#### AUTHOR CONTRIBUTIONS

SS, MY and YW conceived and designed the study, and critically revised the manuscript. SS, YY and HJ performed the experiments, analyzed the data. SS and YY contributed to writing the manuscript. All authors read and approved the final manuscript.

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