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RESEARCH ARTICLE

Effects of JAK2 / STAT3 Signaling Pathway Activation on Intracellular Survival of *Brucella*

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ABSTRACT

Brucellosis is an important zoonotic pathogen of worldwide distribution that causes disease in both humans and animals. *Brucella* can weaken the immune ability of host cells and its pathogenesis depends on its ability to replicate intracellularly and inhibit host cell apoptosis. The JAK2/STAT3 pathway is known to regulate various biological functions, to include cell differentiation, cell death, and apoptosis. However, the role of this pathway in the survival of virulent or low virulent *Brucella* strains in macrophages remains largely uncharacterized. In this study, the JAK2/STAT3 pathway was found to be activated by both smooth *Brucella abortus* 2308 (*B. abortus*; virulent) and rough *B. abortus* RB51 (low virulent), but only *B. abortus* RB51 significantly induced JAK2 and STAT3 phosphorylation in a time-dependent manner. Furthermore, in *B. abortus* RB51, JAK2/STAT3 phosphorylation was inhibited by AG490 (inhibitor of JAK2/STAT3 pathway) in a dose-dependent manner and *B. abortus* RB51-mediated apoptosis and cytokines (IL-6, TNF-α) expression were also inhibited. These findings suggest that JAK2/ STAT3 pathway is important to intercellular survival during a *B. abortus* RB51 infection, but does not appear to play an apparent role in *B. abortus* 2308 survival.

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INTRODUCTION

Brucella is a facultative intracellular bacterium that infects animals and humans (Waqas *et al.*, 2016). It can be found in most countries around the world and contributes to heavy economic losses and human infections (Bolle *et al.*, 2015). *B. abortus* has a complex mechanism for evading the host immune system and also has unique virulence characteristics. Virulent strains are able to resist immune cell cytotoxicity and interfere the innate and adaptive immune responses, thereby enabling host immune evasion and persistent infection. The *B. abortus* stable rough, attenuated mutant vaccine strain RB51 was derived from *B. abortus* strain 2308, a smooth virulent strain. However, there is no effective and safe vaccine for prevention of brucellosis in animals and humans (Zhang *et al*., 2016), and the physiopathology of brucellosis also remains poorly understood. Thus, exploring the specific pathogenic and immune escape mechanism of *Brucella* infection may be the main research direction in the future.

JAK/STAT pathway is one of the major intracellular signal transduction pathway, which also plays a critical role in many biological processes (Chen *et al.*, 2014). This classical signaling pathway is composed of a family of Janus kinase (JAK) proteins (JAK1, JAK2, JAK3 and TYK2) and Signal Transducer and Activator of Transcription (STAT) proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) (Mughal *et al.*, 2014). Research has shown that this pathway increases the secretion of Th2 cytokines, which not only induced cell proliferation, differentiation, apoptosis, and cell death, but also exhibited a specific and pleiotropic effect on the immune system (Yang *et al.*, 2008). Additionally, JAK2/STAT3 pathway activation promotes apoptosis and leads to cell survival and growth both *in vitro* and *in vivo* (Park *et al*., 2016). While this pathway has been well characterized in several tumor types, little is known regarding its role in the infection of pathogenic bacteria, especially intracellular bacteria such as *Brucella*. Current *Brucella* studies have focused on its infection of macrophages and its ability to suppress apoptosis (Ozkaraca *et al*., 2016). However, the role of the JAK2/STAT3 pathway in *Brucella* infection still needs further verification and exploration.

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The aim of this study was to explore the relationship between *B. abortus* survival and JAK2/STAT3 pathway activity in host macrophages following infection with either virulent or low virulent *B. abortus* strains. This study demonstrated that RAW264.7 macrophages infected with *B. abortus* RB51 (low virulent) activate the JAK/STAT3 pathway, thereby inhibiting apoptosis and pro-inflammatory cytokine expression and affecting intracellular survival of *B. abortus* RB51, but does not appear to play an apparent role in *B. abortus* 2308 survival.

MATERIALS AND METHODS

Culture of RAW 264.7 macrophages and *Brucella*: Macrophages were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% Fetal bovine serum (FBS) at 37°C and 5% CO_2. After culturing for 24 h, cells were infected with either *B. abortus* 2308 (virulent) or *B. abortus* RB51 (low virulent). *B. abortus* 2308 and *B. abortus* RB51 were expanded at 37°C on tryptic soy agar (USA), with rotary shaking at 200 rpm for 18 to 24 h. All of the infection experiment was completed in P2 (Physical Containment Level 2) Laboratory (Key Laboratory of Zoonosis, Shihezi university, Xinjiang, China).

MTT assays: The cells were cultured in 96-well culture plates (10^4 cells/well) and were washed with phosphate-buffered saline (PBS). The cells within the experimental groups were pre-treated with 5, 10, 15, 20, or 25μM of AG490 and 0.1% dimethyl sulfoxide (DMSO) for the control group. The cells were cultured at 37°C and 5% CO_2 prior to the cell viability assessment via a MTT assay (Sigma). MTT was added and the cells were incubated for 4h at 37°C. The supernatants were then removed and each well was added with 100μl of DMSO. The plates were quantified at OD_{570}.

RAW264.7 macrophages infected with *Brucella*: To examine the effect of the JAK2 inhibitor AG490 on the virulent and low virulent *Brucella* infection. Cells were cultured in 6-well plates (2×10^6 cell/well), pre-treated with AG490 (5μM, 10μM, or 15μM) for 1 h and then infected with either *B. abortus* 2308 or *B. abortus* RB51 (2×10^8 cell/well, MOI:80:1) by McFarland turbidity standards. After 1 h post infection, cells were washed twice and then incubated for 45 min in DMEM with gentamicin (30 μg/mL) to kill any remaining adherent extracellular bacteria. The remaining infected cells were washed with PBS and lysed at 4, 12, 24, 48 h. The lysates were then centrifuged for 15 min at 12,000g and 4°C, and the live *B. abortus* were enumerated by plating on TSA plates for CFU (Colony-Forming Units) test. At 12 h, the supernatants were collected, the levels of IL-6, IL-1β and TNF-α were examined using an ELISA Quantikine Mouse kit (R&D Systems).

Western blot analysis: To examine p-JAK2 and p-STAT3 (Santa Cruz Biotechnology, USA) expression in macrophages, cells lysates were obtained by incubating with ice cold RIPA lysis buffer containing PMSF for 15 min after 4, 12, 24, 48 h post infection. The samples were then centrifuged at 12,000g for 25 min at 4°C and the supernatant was collected. The protein samples were then boiled for 10 min and 25μL sample was loaded onto a 12% SDS-PAGE. The proteins were then transferred onto a NC (nitrocellulose) membrane (Bio-Rad) at 200mA for 1h. The NC membrane was blocked with 2% BSA in temperature shaker for 1.5 h at 37°C, followed by washing three times with TBST buffer for 25 min. The membranes were then incubated with primary antibodies, p-JAK2 (1:200), p-STAT (1:250), or β-actin (1:5,000), overnight at 4°C. Following the incubation, the membrane was incubated with goat anti-rabbit IgG (Sangon Biotech, China) for 1h and visualized with diaminobenzidine (DAB).

Apoptosis detection: Cells were pre-treated with AG490 (15μM) for 1h. Infection methods and procedures were performed as described above. Apoptotic rates were analyzed using an Annexin V-EGFP Apoptosis Detection kit (Bestbio, Shanghai, China). Annexin V/PI double-positive staining and fluorescence intensity measurements were performed on RAW264.7 cells. The rates of apoptosis were determined using a FACS flow cytometer (BD iosciences).

Statistical analysis: One-way ANOVA, Student's t-tests and Student-Newman-Keuls (SNK) test were used for comparisons among different groups, with the aid of GraphPad Prism and SPASS.

RESULTS

The detection of JAK2/STAT3 signaling pathway activity by Western Blot at various times post-infection: To determine whether the virulent and low virulent strains of *Brucella* modulate the JAK2/STAT3 pathway, JAK2 and STAT3 phosphorylation levels were examined at 0, 4, 12, 24, 48 h post-infection (multiplicity of infection (MOI): 80: 1=bacteria: cell; 0 h=control group). The JAK2/STAT3 pathway activation varied between *B. abortus* 2308 (virulent) and *B. abortus* RB51 (low virulent), with a high degree of expression seen in *B. abortus* RB51 relative to the virulent strain (Fig. 1). Furthermore, the Phosphorylation expression levels of JAK2 and STAT3 were significantly different between the 2308 and RB51 strains at 12h and 48h, with the highest expression levels seen at 12h. These data showed that *B. abortus* RB51 (low virulent) infection enhanced transient JAK2 and STAT3 phosphorylation.

Concentration-dependent manner affected cell viability by AG490: Cell viability was examined via MTT assay, with RAW264.7 cells pre-treated with AG490 (JAK2/STAT3 inhibitor) for 24h at five different concentrations. While the results showed that AG490 inhibited cell viability in a concentration-dependent manner, no significant differences were noted between AG490 concentrations of 5 and 15μM relative to the DMSO control group (*$P<0.05$, Fig. 2).

Effects of the AG490 inhibitor on JAK2/STAT3 signaling pathway activity: To further investigate whether JAK2 and STAT3 phosphorylation is regulated

by AG490 in *Brucella*-infected cells, cells were pre-treated with AG490 at 4 different concentrations and then infected with either *B. abortus* 2308 or *B. abortus* RB51 (MOI: 80:1). At 12h post-infection, AG490 inhibited JAK2 and STAT3 phosphorylation in a dose-dependent manner in *B. abortus* RB51 treated cells, and the largest decrease in JAK2 and STAT3 phosphorylation was seen at a concentration of 15μM (Fig. 3), but no obvious trend was seen following *B. abortus* 2308 infection (data not shown). These results suggest that *B. abortus* RB51 indeed activates JAK2 / STAT3 pathway.

Inhibition of JAK2/STAT3 affects the cytokine response in *B. abortus* RB51-infected cells: Previous studies have indicated that altered cytokine levels are associated with brucellosis in both humans and animals (Demirdag *et al*., 2003). Moreover, JAK and STAT were found to regulate these cytokines. Thus, the ability of JAK2/STAT3 signaling to modulate IL-6 and TNF-α production was examined in *Brucella*-infected macrophages. Cells were pre-treated with various concentrations of AG490 (5, 10 or 15μM) and cytokine levels were examined via ELISA. In comparison to *B. abortus* 2308 infected cells, *B. abortus* RB51-infected cells produced significantly lower levels of IL-6 and TNF-α at AG490 concentrations of 10 and 15 μM ($P<0.05$; Fig. 4). These results suggest that AG490 could affect Th1 and proinflammatory responses in *B. abortus* RB51-infected cells.

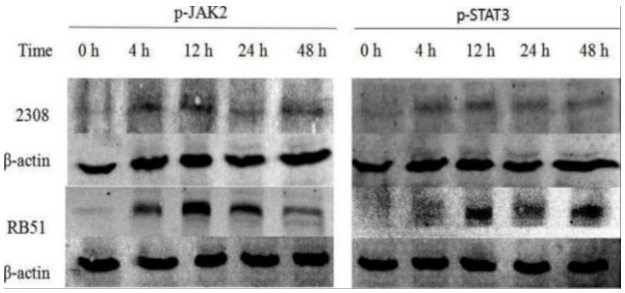


Fig. 1: Represent Western Blot examining JAK2/STAT3 signaling following *Brucella* infection at various times post-infection Western blot analysis of p-JAK2 and p-STAT3 in RAW264.7 macrophages infected with either *B. abortus* 2308 or *B. abortus* RB51 for 4, 12, 24 and 48h. β-actin levels are shown as a loading control.

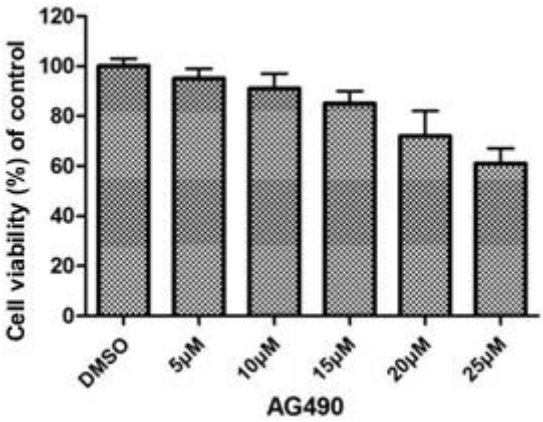


Fig. 2: RAW264.7 cell viability was inhibited following AG490 treatment in a concentration-dependent manner. AG490 treatments were compared with the DMSO and statistical significance indicated (*$P<0.05$).

Inhibition of JAK2/STAT3 effects apoptotic responses in *Brucella*-infected cells: Previous research has indicated that virulent *Brucella* inhibits apoptosis in cells, which was beneficial to intracellular survival of *Brucella* (Pei *et al*., 2014). And it knows that JAK2/STAT3 inhibitor induces apoptosis of cell lines, then to investigate whether this pathway regulates inflammatory cell apoptosis. The JAK2/STAT3 pathway was inhibited following AG490 pre-treated and then cells were infected with either *B. abortus* 2308 or *B. abortus* RB51 (MOI: 80:1) (Fig. 5). At 12h post-infection, cellular apoptotic rates were examined via flow cytometry. No significance difference in apoptotic rate was noted between the 2308-infected AG490-treated cells (9.84%) and 2308-infected cells (10.60%), but the apoptotic rate of the RB51-infected cells (27.76%) was significantly higher than that of RB51-infected AG490-treated cells (19.34%) ($P<0.05$). Thus, the results showed that the virulence of *Brucella melitensis* can affect the regulation of the JAK2/STAT3 pathway, and it is conceivable to suggest that JAK2/STAT3 pathway plays an important role in apoptosis in *B. abortus* RB51-infected cells.

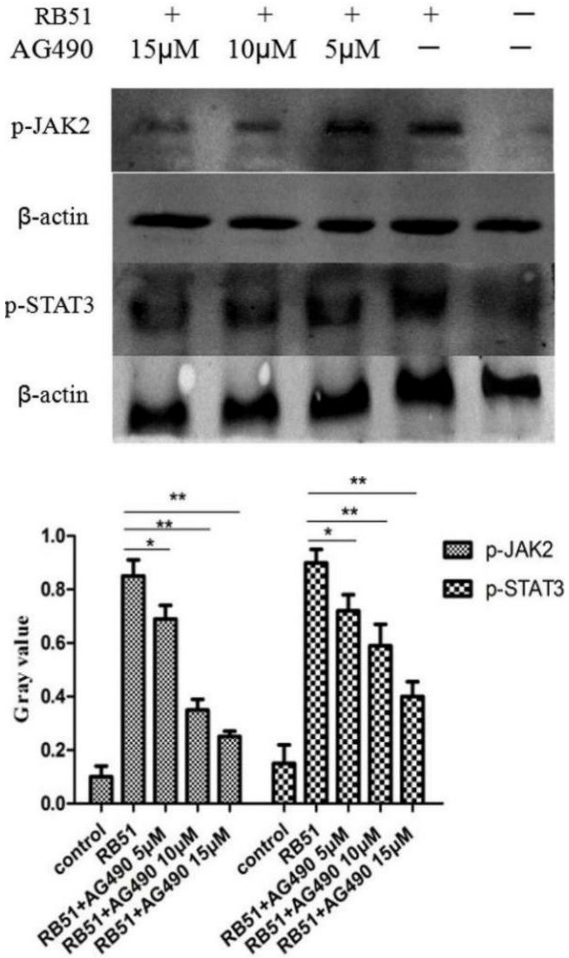


Fig. 3: Effects of different AG490 inhibitor concentrations on JAK2/STAT3 signaling activity. RAW264.7 macrophages were pre-treated with AG490 and infected with *B. abortus* RB51 (MOI: 80:1) for 12h, with no inhibitor pre-treatment used as a control. JAK2 and STAT3 phosphorylation levels were analyzed via Western blotting, with β-actin used as a loading control. Gray value graphs of JAK2 and STAT3 in cells pre-incubated with AG490 inhibitor (15, 10 or 5μM) and infected with *Brucella* are displayed. Statistical significance is indicated by *$P<0.05$ or **$P<0.01$.

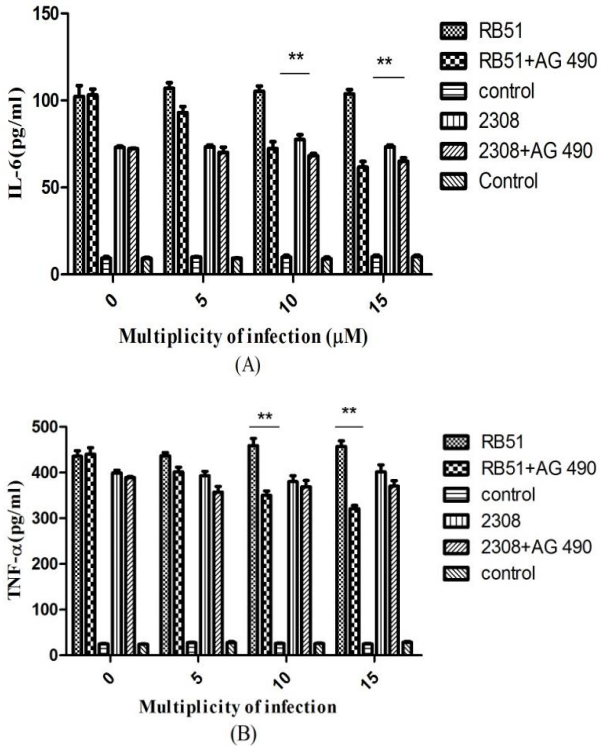
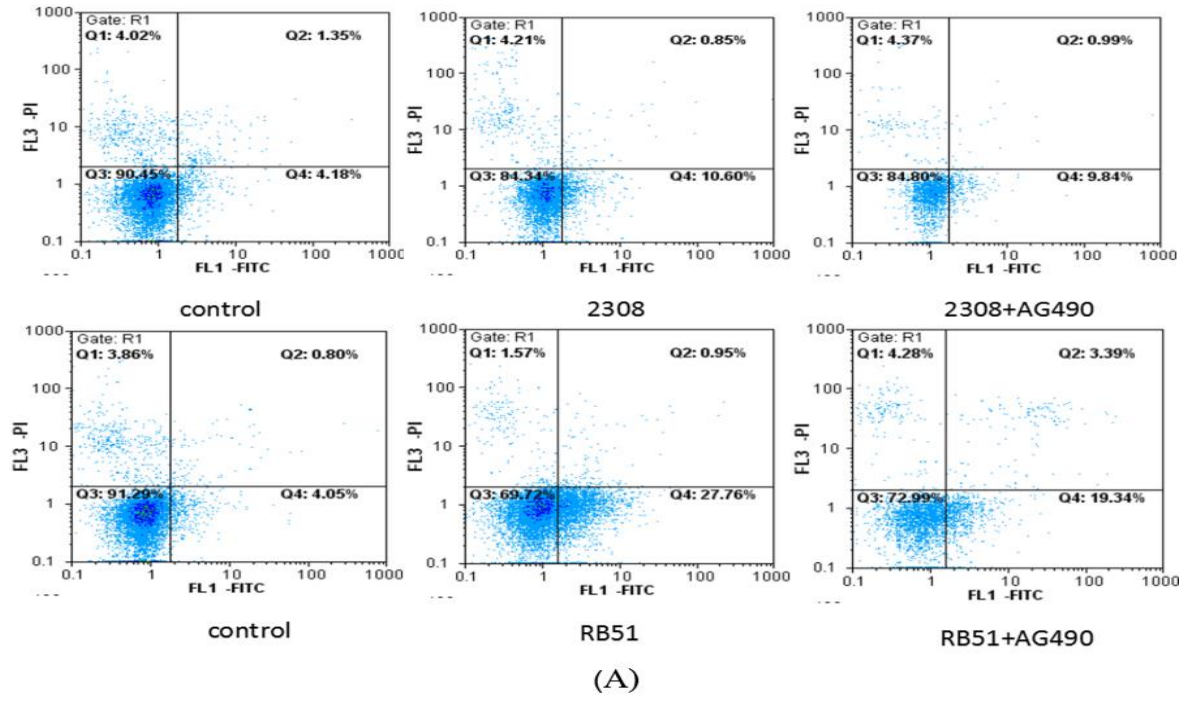


Fig. 4: The effect of inhibitor treatment on cytokine secretions examined via ELISA. Supernatants from *Brucella*-infected AG490 treated cells (5, 10 or 15μM) were collected at 12h post-infection and IL-6 (A), TNF-α (B) secretion were measured using an ELISA. Significant differences between the two strains are indicated by *P<0.05 or **P<0.01. Results are expressed as the mean ± standard deviation of the absorbance values at 450 nm (OD450)

The JAK2/STAT3 pathway effects *Brucella* intracellular survival in RAW264.7 macrophages: *Brucella* does not possess a wide array of virulence factors, thus its virulence is dependent on its ability to invade and survive in host cells. Therefore, we next investigated whether JAK2/STAT3 inhibition affected the intracellular survival following an infection with either *B. abortus* 2308 or *B. abortus* RB51 was examined (MOI: 80:1). For the intracellular replication study, the number of CFU was counted at 4, 12, 24, 48h post-infection, we found that the number of *B. abortus* 2308 intracellular replication was increased gradually inside macrophages, and opposite results were obtained on *B. abortus* RB51 infection. In addition, at 12, 24h post infection, a significant increase was observed in the bacterial CFU representing *B. abortus* RB51 replication inside of the AG490-treated cells compare with that of the control macrophages (AG490-untreated). (P<0.05; Fig. 6). Furthermore, the JAK2/STAT3 (AG490) inhibition had no effect on survival of *B. abortus* 2308 inside RAW264.7 macrophages (Fig. 6). These results show that inhibition of the JAK2/STAT3 pathway benefits *B. abortus* RB51 intracellular replication and survival in RAW264.7 macrophages.

DISCUSSION

Brucellosis is becoming a worldwide epidemic within a certain scope (Zhong *et al*., 2016). *Brucella* not only has the ability to resist phagocytosis, but can also prevent antigen-specific cell recognition (Rodríguez *et al*., 2017). This ability contributes to *Brucella* survival and reproduction and can often lead to a chronic infection. *B. abortus* 2308 has complete lipopolysaccharides (LPSs), while *B. abortus* RB51 is an attenuated rough, lipopolysaccharide O antigen-deficient mutant (Mariani *et al*., 2000). JAK and STAT proteins are actively involved in cellular survival, proliferation, differentiation, and apoptosis (Truong *et al*., 2017). However, whether JAK2/STAT3 signaling contributes to intracellular survival in virulent or low virulent *Brucella* strains remains unclear. This study demonstrates that *B. abortus* RB51 activates JAK2/STAT3 pathway in a time-dependent manner, but *B. abortus* 2308 was found to not significantly affect the activation of this pathway. In addition, the JAK2/STAT3 pathway is essential for intracellular survival delay in RB51-infected macrophages.



(A)

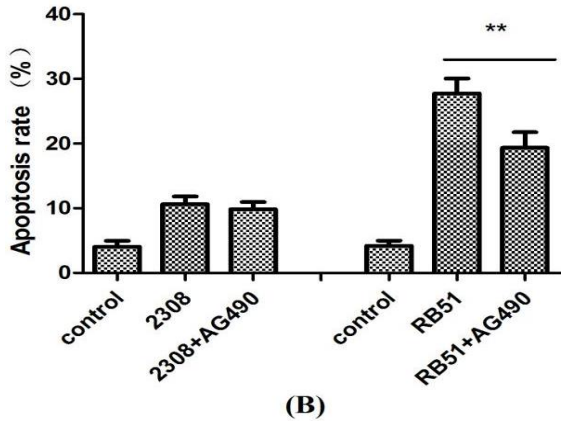


Fig. 5: Analysis of macrophage apoptosis induced by *B. abortus* 2308 and *B. abortus* RB51 using Annexin FITC/PI. (A) Flow cytometry analysis of apoptosis in *Brucella*-infected cells, the infections with or without pre-treatment were then examined. (B) The apoptotic rate of the *Brucella*-infected cells.

Fig. 6: *Brucella* intracellular survival with and without inhibitor pre-treatment. RAW264.7 macrophages were infected with either *B. abortus* 2308 or *B. abortus* RB51 and survival was determined at different times post-infection. The infections with or without pre-treatment were then examined, with significant differences indicated by *P<0.05 or **P<0.01.

Previous studies have confirmed that activated JAKs subsequently phosphorylate and activate STAT proteins, thereby leading to dimerization, nuclear translocation, and DNA binding (Lavecchia *et al*., 2011). AG490, a specific and potent inhibitor of JAK2, has been shown to predominantly inhibit the activation of the JAK2/STAT1 pathway (Fu *et al*., 2017). *Mycobacterium tuberculosis* has also been shown that JAK2 (AG-490) inhibited TNF-α and NO production, caspase 1 activation and apoptosis. Herein, pre-treatment with AG490 showed a dose-dependent inhibition of p-JAK2 and p-STAT3 in *B. abortus* RB51 infected cells, while no obvious trend was noted following *B. abortus* 2308 infection. These findings indicated that the underlying mechanism of *Brucella* infection may involve JAK2/STAT3 activation. Thus, the impact of this activation on cytokine signaling was examined next to gain insight into the host immune response.

Cytokines play a key role in the regulation of the immune response (Bitsue *et al.,* 2017). JAK2-STAT3 pathway is one of the important inflammatory signaling pathways in the immune system, Classical studies suggest that this pathway promotes the secretion of TNF-α and IL-6, resulting in an inflammatory response (Sugaya, 2008), and JAK2/STATs signaling being a common pathway used by many cytokines to regulate target genes relating to cell survival and immune responses in pathological process (Hoyt *et al.,* 2007). Previous studies have indicated that cytokines such as TNF-α, IFN-γ, can regulate the survival of *Brucella* in macrophages during the process of *Brucella* infection (Golding *et al.,* 2001). And virulent *Brucella* inhibits apoptosis in macrophages, which promotes *Brucella* survival (Pei *et al*., 2014). Thus, it is key to know that whether affect the secretion of cytokines and the activity of JAK2/STAT3 pathway in *Brucella*-infected macrophages. In this study, we observed that various inhibitor concentrations were found to affect the expression of the inflammatory cytokines TNF-α and IL-6. Previous studies showed that IL-6 was a key mediator of cell-mediated immunity and could enhance the immune response against infections. TNF-α has been shown to stimulate the amplification of multiple signaling pathways through several cascaded receptor molecules and can also induce cellular apoptosis (Qi *et al*., 2017). The findings presented herein are consistent with these findings and show that JAK2/STAT3 pathway contributes to the immune response strategy in RB51-infected macrophages. Moreover, it can be inferred that the JAK2/STAT3 mediated release of TNF-α and IL-6 may impact the intracellular survival of *Brucella*. JAK2/ STAT3 pathway is an important intracellular signal transduction pathway, which can be activated by various inflammatory factors, growth factors and environmental stress reactions and mediated cell proliferation, differentiation, apoptosis and another signal transmission. After *Brucella* infection, whether it regulates macrophage apoptosis and bacterial intracellular survival and what is the effects of blocking this pathway, these problems have not been reported at home and abroad (Kowshik *et al*., 2014). In my study, JAK2/STAT3 inhibition was found to reduce apoptosis of *B. abortus* RB51-infected macrophages. Additionally, when performing a CFU assay, the JAK2/STAT3 pathway was found to enhance macrophage anti-microbial activity. These findings suggest that JAK2/STAT3 activation decreases *B. abortus* RB51 survival *in vitro*. Furthermore, these findings are consistent with previous studies that have suggested that *Brucella* has the ability to survive and replicate inside of host cells, with pathogen success dependent on the production of various virulence factors that affect phagocytosis, cytokine secretion and apoptosis (Vrioni *et al*., 2008). Moreover, previous studies have shown that an intracellular *Brucella* infection can inhibit macrophage apoptosis (Zhang *et al*., 2016). In agreement with these findings, this study shows that JAK2/STAT3 signaling can regulate the host cell immunity and inhibit the survival and replication of *Brucella*.

Additionally, previous studies have found a causal correlation between LPS and *Brucella* virulence, with the LPS integrity correlating to survival (Zhao *et al*., 2017). In the present study, *B. abortus* 2308 weakly activated the JAK2/STAT3 pathway when compared to *B. abortus* RB51. Moreover, in *B. abortus* 2308-infected macrophages, JAK2/STAT3 inhibition did not alter TNF-α or IL-6 expression. These findings confirm that the LPS on the surface of *B. abortus* 2308 did not activate the JAK2/STAT3 pathway completely and that the LPS O-chain may play an important role in *Brucella* infection. However, further studies are required to validate this hypothesis.

Conclusions: In this study, we demonstrated that JAK2/STAT3 phosphorylation/activation is increased in *B. abortus* RB51-infected macrophages. Furthermore, the activation of this pathway would presumably promote apoptosis, and its inhibition enhances Th1 cytokine expression and proinflammatory molecules. While these findings suggest that *B. abortus* RB51 induces JAK2/STAT3 activation, cellular signal transduction is complex and further exploration into the interaction between each of the signaling pathways associated with a *Brucella* infection is still required. Overall, the results of this study contribute to the further unraveling of the pathogenic mechanisms of virulent and low virulent *Brucella* and may aid in the development of new drugs against *Brucella* infections.

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Authors contribution: JY, YW and CC conceived and designed the project and study. TL and JZ executed the experiment and analyzed the sera and tissue samples. JX analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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