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Research Article

The Role of JAK2/STAT3 Signaling Pathway Regulation in Macrophage Apoptosis During *Brucella* M5-90 Infection

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Abstract

Brucellosis is serious zoonotic disease affecting both animals and humans. *Brucella* inhibits the apoptosis of host macrophages. And the JAK2/STAT3 pathway regulates various cellular physiological activities. However, the association between *Brucella*-mediated inhibition of macrophage apoptosis and regulation of the JAK2/STAT3 pathway is unclear. In the current study, We tested the the activation of JAK2/STAT3 pathway and evaluated its function during *Brucella* M5-90 infection in cells. The result was found that infection with *Brucella* M5-90 activated the JAK2/STAT3 pathway and induced phosphorylation of both JAK2 and STAT3 in a time-dependent manner. JAK2 and STAT3 phosphorylation were inhibited by AG490 in a dose-dependent manner. Inhibition of the JAK2/STAT3 pathway with AG490 significantly induced proinflammatory responses, macrophage apoptosis at the transcriptional and protein levels, as well as intracellular survival and replication of *Brucella* M5-90. This study indicates that the JAK2/STAT3 pathway plays a major role in macrophage apoptosis induced by infection with *Brucella* M5-90. In addition, TNF-α plays a major role in the regulation of the JAK2/STAT6 pathway during *Brucella* M5-90 infection. The above information may help to unravel the pathogenic mechanism of Brucella infection.

Keywords: *Brucella, JAK2/STAT3 pathway, Apoptosis, Infection, AG490*

Brucella M5-90 Enfeksiyonu Süresince Makrofaj Apoptozisinde JAK2/STAT3 Uyarı Yolağının Rolü

Öz

Brusellozis hem hayvanları hem de insanları etkileyen ciddi zoonotik bir hastalıktır. *Brucella* konakçı makrofajlarının apoptozisini inhibe eder. JAK2/STAT3 yolağı çeşitli hücresel fizyolojik aktiviteleri düzenler. Ancak *Brucella* aracılı makrofaj apoptozisinin inhibisyonu ile JAK2/STAT3 yolağının düzenlenmesi arasındaki ilişki belirsizdir. Bu çalışmada; JAK2/STAT3 yolağının aktivasyonu çalışılarak hücrelerde *Brucella* M5-90 enfeksiyonu boyunca fonksiyonu değerlendirildi. *Brucella* M5-90 ile enfeksiyonun JAK2/STAT3 yolağını active ettiği ve hem JAK2 hem de STAT3 fosforilasyonunu zamana bağlı olarak uyardığı tespit edildi. JAK2 ve STAT3 fosforilasyonu doza bağlı olarak AG490 ile inhibe edildi. JAK2/STAT3 yolağının AG490 ile inhibe edilmesi anlamlı derecede proinflamatory yanıtı, transkripsiyon ve protein seviyesinde makrofaj apoptozisini ve *Brucella* M5-90'nın hücre içi hayatta kalma ve replikasyonunu uyardı. Bu çalışma *Brucella* M5-90 enfeksiyonu ile oluşturulan makrofaj apoptozisinde JAK2/STAT3 yolağının önemli bir rol oynadığını göstermiştir. Ayrıca, *Brucella* M5-90 enfeksiyonu süresince JAK2/STAT6 yolağının regülasyonunda TNF-α önemli rol oynar. Yukarıda ifade edilen bulgular *Brucella* enfeksiyonunun patogenezini açığa çıkarmada faydalı olabilir..

Anahtar sözcükler: *Brucella, JAK2/STAT3 yolağı, Apoptozis, Enfeksiyon, AG490*

INTRODUCTION

Brucella abortus are Gram-negative and facultative intracellular pathogens affecting humans and animals and can enter various cell types during infection [1]. *Brucella* penetrates the nasal cavity, oral cavity, and/or pharynx mucous membrane and is phagocytosed by host macrophages, in which they can survive and replicate.

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Brucellosis is a zoonotic disease, which is difficult to diagnose. Infection in humans causes fever, hyperhidrosis and arthralgia along with other symptoms. During *Brucella* infection, bacteria preferentially invade host macrophages or trophoblast cells. Although bacterial invasion generally activates the body's specific immunity and natural immunity to promote the lethality of immune cells, *Brucella* strains have developed strategies for evading these cells [2]. *Brucella* resides and replicates within the endoplasmic reticulum of cells, even regulating the apoptosis of these host cells, thus weakening the killing ability and phagocytosis of immune cells. Studies have shown that Tumor Necrosis Factor (TNF-α) is important for inducing specific immune responses against a series of intracellular infections [3]. Furthermore, it has been proven that TNF-α-mediated apoptosis plays a role in pathologies related to chronic inflammation and autoimmune diseases [4]. Therefore, understanding the pathogenesis, survival and replication of *Brucella* in macrophages, as well as the immune mechanisms involved in combating bacterial infection, are vitally important for treating chronic infection with *Brucella*.

The JAK2/STAT3 signal transduction pathway is an important intracellular signal transduction pathway and is a common pathway for a variety of cytokines and growth factors. The JAK2/STAT3 pathway plays an important role in cell proliferation, differentiation, apoptosis and immune regulation [5]. Additionally, research [6] has shown that at present, there are few studies of the regulatory mechanisms involved in host cell immune pathways after intracellular infection. The current study examines whether the JAK2/STAT3 pathway plays a role in the regulation of host cell apoptosis and survival of intracellular bacteria survival during *Brucella* infection.

The inhibitor, AG490, was used to inhibit JAK2 activity [7] in macrophage model of mice infected with *Brucella* M5-90 to determine the relationship between the JAK2/STAT3 signal pathway and apoptosis of infected macrophages. Western blot, ELISA, RT-PCR and CFU assays were used to detect JAK2 and STAT3 phosphorylation, cytokine secretion, changes in apoptosis and intracellular survival of *Brucella*, respectively. The aim of this study was to explore the regulatory role of the JAK2/STAT3 signal pathway in macrophage apoptosis induced by *Brucella* and to determine whether the JAK2/STAT3 signal pathway may be useful in developing treatments for *Brucella* infection.

MATERIAL and METHODS

Cell Culture

RAW264.7 macrophages were obtained from the China Academy Typical Culture Preservation Committee Cell Library (Shanghai, China). RAW264.7 cells were cultured in 12-well plates in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at a concentration of 1×10^6 cells per well and cultured overnight at 37°C in humidified 5% CO_2.

Brucella Infection

To determine whether AG490 (JAK2/STAT3 pathway inhibitor) affects the activity of the JAK2/STAT3 signaling pathway and the expression of apoptosis related proteins in macrophages, RAW264.7 cells were pre-treated with different concentrations of AG490; control cells were not treated with inhibitor. RAW264.7 macrophages were infected with *Brucella* M5-90 at a multiplicity of infection (MOI) of 100:1. The cells were incubated for 1 h at 37°C, washed twice with phosphate-buffered saline (PBS), and then incubated in DMED, supplemented with 25 μg/mL gentamicin, for 45 min to kill remaining extracellular and adherent *Brucella* M5-90. The infected cells were washed three times with ice cold PBS and new culture medium was added. At 2, 4, 8, 12 and 24 h post-infection, one group of cells were lysed with RIPA Lysis Buffer (Beyotime, China), centrifuged at 12000 rpm for 20 min at 4°C and the protein supernatant used for analysis of JAK2, STAT3 phosphorylation by Western blot, another group of cells was lysed with 0.1% tritonX-100 for mRNA transcriptome analysis of JAK2, STAT3, Bax, Bcl-2 and a third group of cells were used to detect the TNF-α level in the supernatant using an ELISA Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). All samples were stored at -80°C.

MTT Assay

RAW264.7 cells were cultured in 96-well plates at 37°C under humidified 5% (vol/vol) CO_2 and then treated with 10, 20, 40, or 60 μM AG490 (treated groups) or 0.1% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Inc.) (control group). Cells were incubated in humidified 5% CO_2 at 37°C for 16-48 h to assess cell viability by MTT assay. A 20 μL volume of MTT solution (0.5% MTT) was added to each well and the cells were incubated for 4 h. The culture supernatants were removed. DMSO (150 μL) was then added to each well and the wells were shaken at low speed for 10 min. The optical density (OD) of each well was measured at 490 nm by the ELISA reader (or a microplate reader).

Western Blot Assay

Extracted protein was boiled for 10 min at 100°C and 20 μL samples were subjected to 15% SDS-PAGE. The protein samples were electrophoretically transferred onto a NitroBind nitrocellulose membranes (Bio-Rad; 0.45-μm) at 200mA for 1 h. The membranes were then blocked with Non-protein blocking solution (Shenggong, China) for 1 h and washed three times with TBST buffer (100mM trus-HCl; 150mM NaCl; 0.05% Tween 20, pH 7.2). The membranes were then incubated overnight at 4°C with a 1/1500 dilution of specific primary anti-rabbit pJAK2 (sc-34479), pSTAT3 (sc-56747), Bax(sc-20067), or Bcl-2 (sc-56015), caspase-3(sc-136219) (Santa Cruz Biotechnology, USA) antibodies.

The membranes were subsequently incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (EarthOx Life Sciences, USA) for 1 h at 37°C and the bound conjugate was visualized using HRP-3, 3′-Diaminobenzidine (DAB) Substrate Solution (Thermo Fisher Scientific, USA).

Quantitative Real-Time PCR Analysis

As above, RAW264.7 cells were treated with AG490 (20 μM) for 1 h and then infected with *Brucella* M5-90. Cells were then washed three times with PBS at 4, 8, 12, or 24 h post-infection. TRIzol (1 mL) was added to each well, and passing the cell lysate several times. Total RNA was extracted with AMV (Avian Myeloblastosis Virus) reverse transcriptase (Takara, Tokyo, Japan) following the manufacturer's instructions. Real time quantitative PCR was used to validate cDNA using a Light-Cycler 480 (Roche, Switzerland). The PCR reaction system were as follows: Total 20 μL, SYBR premix E_xTaq 10 μL, cDNA model 2.0 μL, Up Primer 0.4 μL, Down-Primer 0.4 μL, dH_2O 7.2 μL. And the reaction conditions were as follows: 5 min at 95°C followed by 40 cycles at 95°C for 30 sec, 55°C for 30 min, and 72°C for 5 min. GAPDH was used as the reference. The primers for Bax were sense 5′-GACTTCUCUCGTCGCTACCG-3′ and anti-sense 5′-ACAATCCTCCCCCAGTTCAC-3′, the primers for Bcl-2 were sense 5′-TTCTTTGAGTTCGGTGGGGTC-3′ and anti-sense 5′-TGCATATTTGTTTGGGGCA

GG-3′ and the primers for GAPDH were sense 5′ CTGCCC AGAACATCATCCCT3′,and anti-sense 5′ GACACATTGGGGG TAGGAAC-3′.

Apoptosis Assay

Brucella M5-90-infected RAW264.7 cells were incubated with Annexin V and propidium iodide (PI) at 37°C for 20 min using an Annexin V-FLUOS staining kit (BB-410; Bestbio, Shanghai). The rate of apoptosis in the RAW264.7 cells was then detected immediately following incubationvia flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Intracellular Survival Assay

RAW264.7 cells were pre-treated with AG490 (20 μmol/L) for 1 h and then infected with *Brucella* M5-90 at a 100:1 MOI, as described above. The infected cells were then lysed at 4, 8, 12, 24, or 48 h post-infection using 1%Triton X-100. The number of bacteria was enumerated by plating different dilutions of the lysates on TSA plates after several days.

Statistical Analysis

Differences among of experimental groups were analyzed by student's t-test. A P-value of <0.05 was considered statistically significant and a P-value of <0.01 was considered greatly significant.

RESULTS

Inhibitor AG490 Affects Cell Viability in a Concentration-Dependent Manner

Cell viability was determined via MTT assay after RAW264.7 cells were incubated for 48 h with a range of AG490 (JAK2/STAT3 inhibitor) concentrations. AG490 affected RAW264.7 cells viability in a concentration-dependent manner. As shown in *Fig. 1*, there was no obvious effect on RAW264.7 cell viability after treatment with 20 μM AG490.

Brucella M5-90 Activated the JAK2 /STAT3 Signaling Pathway

A Western blot was performed to detect the phosphorylation levels of JAK2 and STAT3 at 2, 4, 8, 12 and 24 h post-infection in order whether *Brucella* M5-90 regulates the activity of the JAK2 /STAT3 signaling pathway. As shown in *Fig. 2A*, *Brucella* M5-90 induced JAK2 and STAT3 phosphorylation from 4 h to 24 h post-infection, but the degree of activation was different; phosphorylation peaked at 12 h post-infection. The difference in phosphorylation (12 h and 24 h) was extremely significant compared with 0 h post-infection ($P<0.01$). The phosphorylation level increased at first and then decreased indicating that infection with *Brucella* M5-90 resulted in improved transient phosphorylation of JAK2 and STAT3 proteins.

Whether AG490 affects phosphorylation of the JAK2/STAT3 signaling pathway in AG490-treated RAW264.7 cells infected with *Brucella* M5-90 was also tested. AG490 inhibited JAK2, STAT3 phosphorylation in a concentration-dependent manner. Phosphorylation of the signaling pathway was almost completely blocked after treatment with 20 μM AG490 *(Fig. 2B)*, further confirming that

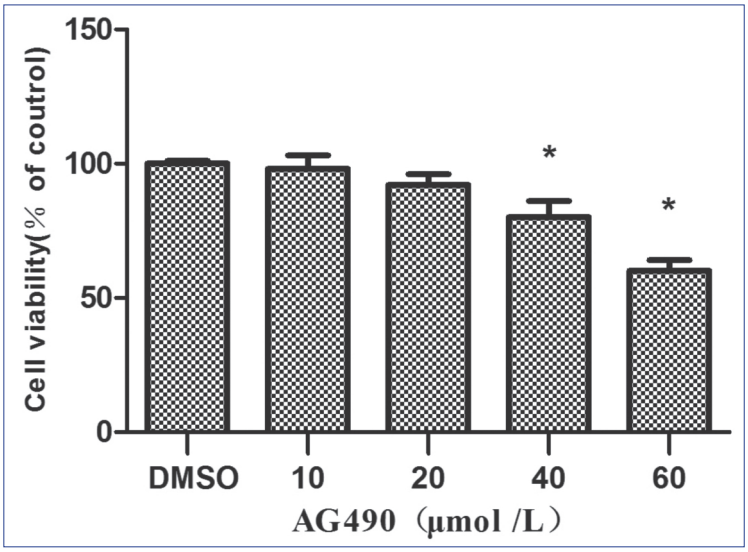


Fig 1. RAW264.7 cell viability was inhibited following AG490 treatment in a concentration-dependent manner. Viability of RAW264.7 cells treated with 40 and 60 μmol/L AG490 was significantly different compared to control cells treated with DMSO. *P<0.05

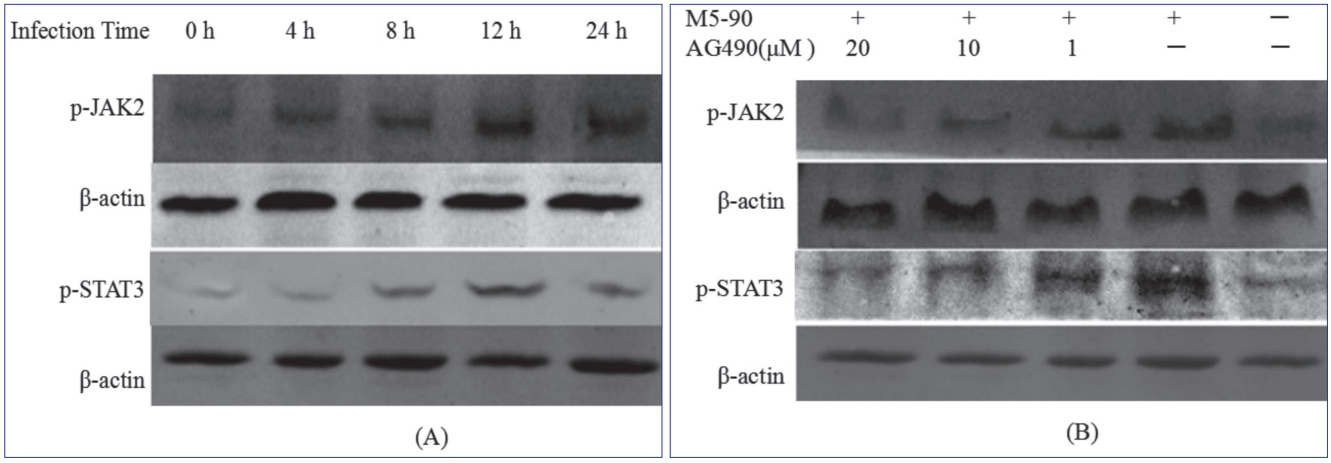


Fig 2. *Brucella* M5-90 activated the JAK2 /STAT3 signaling pathway. (A) Western blot analysis of JAK2 and STAT3 phosphorylation levels from *Brucella*-infected RAW264.7 cells at different time points. (B) Cells were treated with different concentrations of AG490 inhibitor and then infected with M5-90 for 12 h. JAK2 and STAT3 phosphorylation levels were detected by Western blot analysis. β-actin was used as the reference

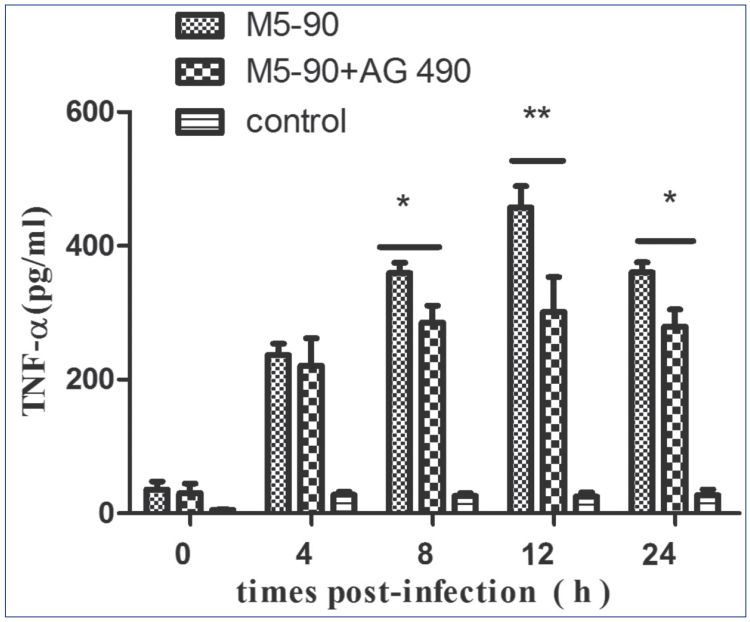


Fig 3. TNF-α levels in AG490-treated RAW264.7 cells and in control cells infected with *Brucella* M5-90. *P<0.05; **P<0.01

Brucella M5-90 activates the JAK2/STAT3 pathway.

The JAK2 /STAT3 Signaling Pathway Regulates the Level of TNF-α in M5-90-Infected Cells

To determine whether the JAK2 /STAT3 signaling pathway regulates the level of TNF-α in M5-90-infected cells, RAW264.7 cells were pre-treated with 20 μM AG490 for 1 h and then infected with *Brucella* M5-90. TNF- α levels were then measured at 0, 4, 8, 12, and 24 h post-infection. The expression of TNF-α gradually increased from 0 h to 12 h and then decreased from 12 h to 24 h. AG490 inhibitor-treated cells produced significantly higher levels of TNF-α compared to control cells (PBS) at 12 h ($P<0.01$); the difference was also significant difference at 8 h and 24 h (*Fig. 3;* $P<0.05$). This indicates that AG490 reduces the expression of TNF-α via inhibiting the activation of the JAK2 /STAT3 signaling pathway.

Analysis of Apoptosis-Related Protein (caspase-3, Bax) Expression in Brucella M5-90-Infected RAW264.7 Cells Treated with AG490

Activation of TNF receptor-1 by TNF-α can induce cell apoptosis and involves consecutive activation of both the NF-κB and caspase pathways [8,9]. Thus, the expressions of Bax and caspase-3 proteins were determined at different time points post-infection to investigate whether the JAK2 /STAT3 signaling pathway is involved in the apoptosis of *Brucella*-infected RAW264.7 cells. Bax is an apoptosis-promoting protein belonging to the B-cell lymphoma 2 (Bcl-2) protein family [10]. Caspase-3 is the most important terminal shear enzyme in the process of apoptosis and can induce apoptosis after caspase-activation [11]. In this study, the expressions of caspase-3 and Bax increased gradually, reaching their highest levels at 12 h, and the expressions of the Bax and caspase 3 proteins were induced after 12

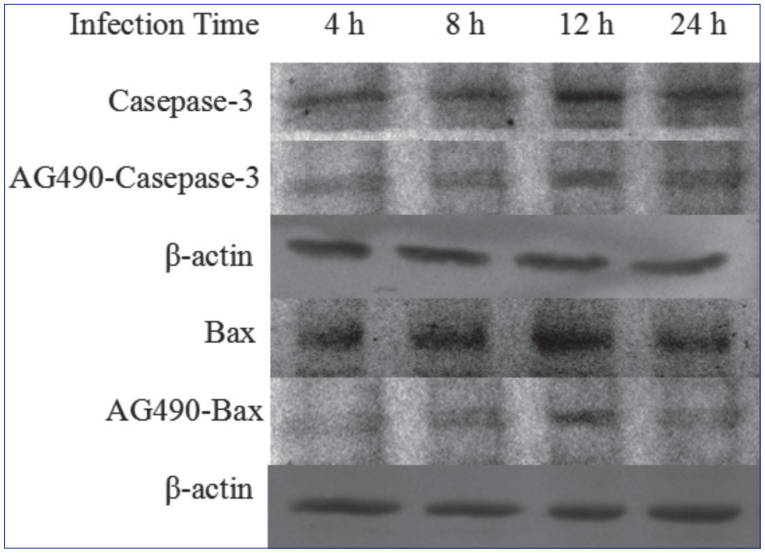


Fig 4. Western blot analysis of apoptosis promoting protein (caspase-3 and Bax) expression in *Brucella* M5-90-infected RAW264.7 cells at different time points

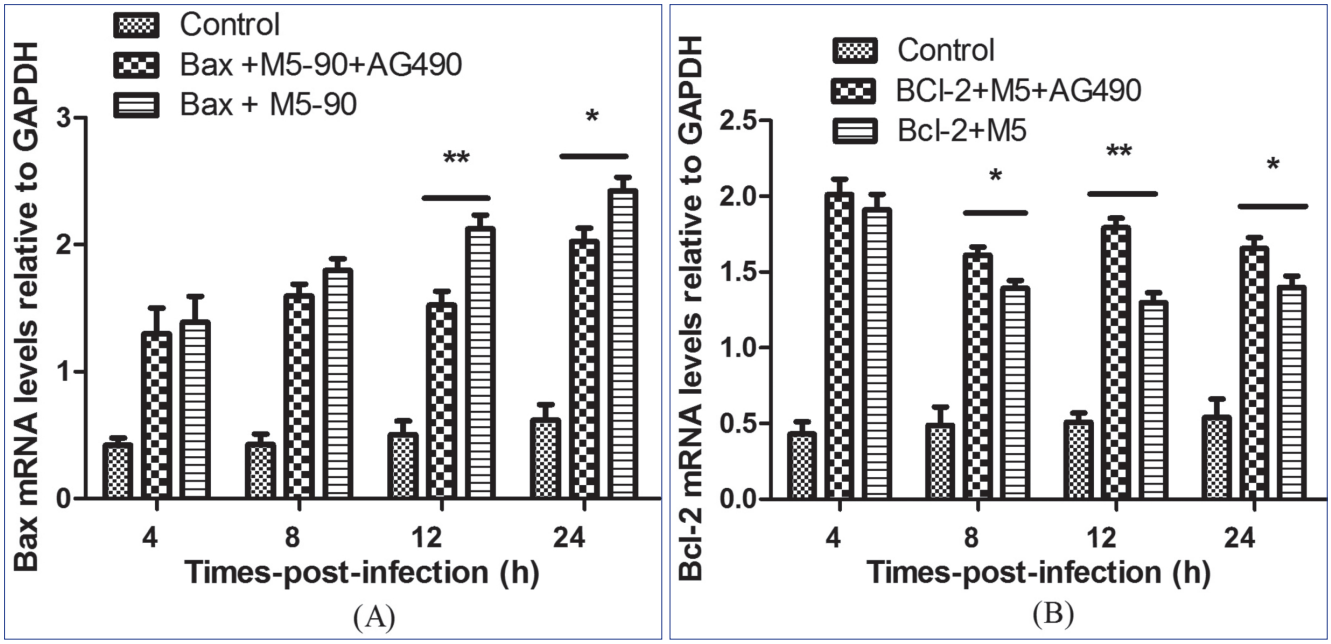


Fig 5. The JAK2/STAT6 pathway significantly affects the mRNA expressions of apoptosis-related genes Bax and Bcl-2 at the transcriptional level. (A) Bax mRNA levels and (B) Bcl-2 mRNA levels at different time points. *P<0.05; **P<0.01

h *(Fig. 4)*. However, exposure of cells to AG490 (20 μM) resulted in a gradual decrease of both Bax and caspase-3 expressions from 4 h to 24 h compared with control cells. The difference in Bax and caspase-3 expression between AG490-treated and control cells was particularly significant at 12 h. These results suggest that AG490 affects secretion of apoptosis related proteins (Bax, caspase-3) in a time-dependent manner indicating a role of the JAK2 /STAT3 signaling pathway in the apoptosis of M5-90-infected cells.

AG490 Affects the mRNA Expressions of Apoptosis-Related Genes Bax and Bcl-2

Bax is involved in the regulation of the apoptosis, Bcl-2 enhances cell activity and survival via the production of Bax- induced cytochrome (Cyt) c, and Cyt c activates the caspase gene in the cytosol, thereby causing cell death or apoptosis [12,13]. Thus, to detect whether the JAK2 /STAT3 signaling pathway affected the expression of apoptosis-related genes on an mRNA level, real-time polymerase chain reaction (RT- PCR) was used to analyze Bax and Bcl-2 mRNA expression levels. As shown in *Fig 5A*, the M5-infected AG490-treated cells transcribed significantly higher mRNA levels of Bax gene than the M5-90-infected cells at 12 h (P<0.01) and 24 h (P<0.05) post infection. In contrast, the result for Bcl-2 shows that the M5-infected AG490-treated cells produced significantly lower mRNA levels of Bcl-2 gene in the M5-infected cells at 8 h (P<0.05), 12 h (P<0.01) and 24 h (P<0.05) post infection *(Fig. 5B)*. These results indicate that AG490 inhibits Bax mRNA levels

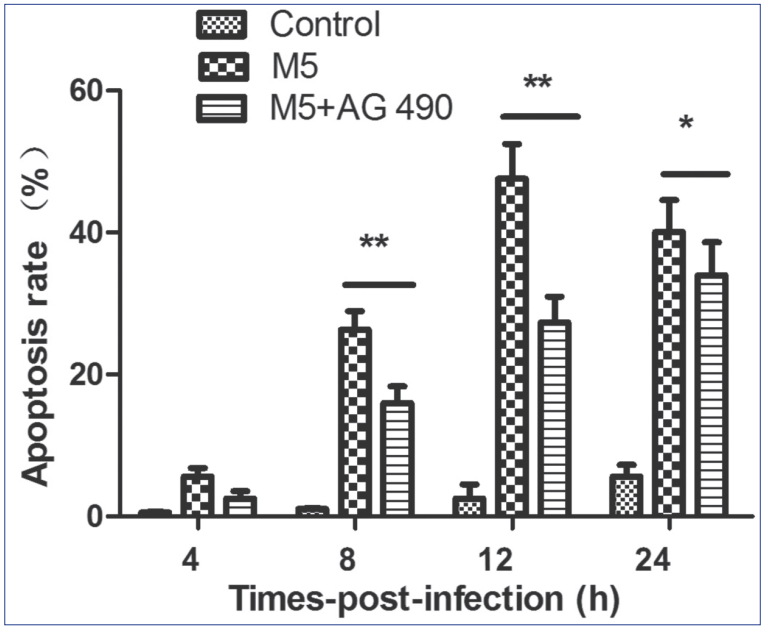
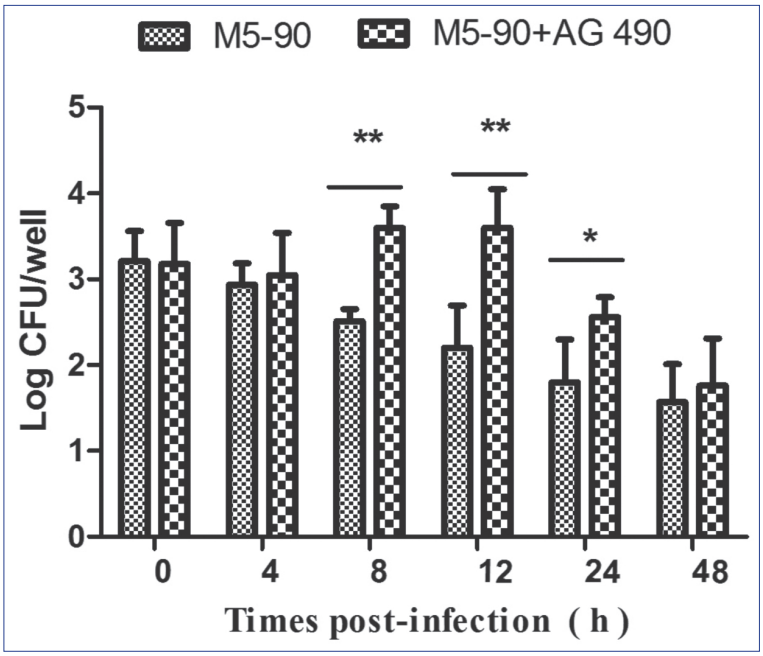


Fig 6. Flow cytometry analysis of apoptosis at different time points using Annexin FITC/PI in RAW264.7 cells infected with *Brucella* M5-90 in the absence or presence of AG490, compared with M5-90-infected untreated control cells.*P<0.05; **P<0.01

Fig 7. Increased *Brucella* M5-90 survival inside RAW264.7 cells with the use of AG490. *P<0.05; **P<0.01



and increases Bcl2 mRNA levels and confirms that the JAK2 /STAT3 signaling pathway regulates apoptosis in M5-90-infected RAW264.7 cells at the transcriptional level.

AG490 Inhibitor of JAK2 /STAT3 Signaling Pathway Induces Apoptotic Rates in M5-90-Infected RAW264.7 Cells

Cells were preincubated with AG490 for 1 h prior to infection with M5-90 for 4 h, 8 h, 12 h, or 24 h; PBS was used as the control. The M5-dependent apoptosis rates were then analyzed using flow cytometry. As shown in *Fig 6,* the apoptotic rate ranged from 2.55% to 33.95% in M5-90-infected AG490-treated cells from 5.64% to 40.09% in M5-90 infected cells and from 0.97% to 5.64% in control cells treated with PBS. The apoptosis rate in M5-90-infected RAW264.7 cells was significantly high than in M5-90 infected cells treated with AG490, particularly at 8 and 12 h (P<0.01). In addition, there was no difference at 4 h post-infection (P>0.05). This result confirmed that inhibition of the JAK2 /STAT3 signaling pathway by AG490 decreased apoptotic rates in M5-90-infected RAW264.7 cells.

The JAK2/STAT3 Signaling Pathway Regulates the Intracellular Survival and Replication of Brucella M5-90

The intracellular survival and replication of *Brucella* M5-90 was measured at six time points in macrophages after a 100:1 MOI. There was no significant difference in the amount of *Brucella* M5-90 intracellular survival inside

AG490-treated macrophages at 0, 4, or 48 h post-infection compared to untreated macroghages. However, bacterial number was higher in AG490-treated macrophages at 8, 12, and 24 h post compared to untreated control cells (1.08-log,1.4-log, 0.76-log; $P<0.05$; *Fig. 7*). These results show that AG490 significantly increased intracellular growth and replication of *Brucella* M5-90 in macrophages, indicating that the JAK2/STAT3 signaling pathway regulates the intracellular survival of *Brucella*. In addition, the results of the above experiment confirmed that the intracellular survival of *Brucella* was inversely proportional to the apoptosis of M5-90-infected cells.

DISCUSSION

JAK2 belongs to the family of protein tyrosine kinases, which mediate the cascade activation reaction of signal protein molecules. JAK2 is activated after cytokines and growth factors bind to its corresponding receptor, thereby phosphorylating STAT3 [14]. STAT3 is an important protein that can be activated by multifunctional cytokines and growth factors. The phosphorylated STAT3 protein regulates various physiological and biological activities [15]. Previous studies have found that the JAK2/STAT3 pathway not only closely affects cell proliferation, differentiation and apoptosis, but also more importantly, it is also involved in mediating the inflammatory and immune responses during the process of disease development [16,17]. AG490, an inhibitor of JAK2, can directly inhibit Osteopontin (OPN)-induced nuclear localization and DNA binding activity of STAT3 indicating that the JAK2 protein is involved in this process [18]. Mycobacterium tuberculosis is an intracellular bacterium and activation of the JAK2/STAT3 pathway can regulate expression of immune factors after human cells are infected. Therefore, the JAK2/STAT3 pathway may regulate cell apoptosis and affect the survival of *Brucella* in macrophages similar to tuberculosis, since *Brucella* is also an intracellular bacterium. In this study, it was confirmed that *Brucella* M5-90 infection activates the JAK2/STAT3 signaling pathway and that the activation of this signaling pathway regulates the apoptosis of the RAW264.7 cells and proinflammatory reactions, thereby affecting the intracellular survival of *Brucella* M5-90.

Brucella initially infects macrophages, which are a first line of defense in the immune system. Macrophages, as immunomodulatory cells, can rapidly kill pathogenic bacteria that invade the body [19]. However, *Brucella* developed a strategy to escape being killed by macrophages [20]. Recent research has indicated that the JAK2/STAT3 pathway plays a crucial role in immune regulation and apoptosis [21]. In this study, the phosphorylation levels of JAK2 and STAT3 increased in RAW264.7 cells infected with *Brucella* M5-90. Therefore, inhibition of the JAK2/STAT3 pathway may affect the transcription of related immune factors when *Brucella* invades the macrophages. To test this hypothesis, cells were treated with AG490 (an inhibitor as described above). It was found that different concentrations of AG490 affected activation of the pathway to varying degrees and that AG490 substantially reduced the expression of TNF-α. TNF-α induces cells apoptosis after engaging TNF-α receptor-1 by activating caspases in the death receptor pathway [8] suggesting that the JAK2/STAT3 pathway triggers macrophage apoptosis via TNF-α. Other studies have also confirmed that Bcl-2 affects TNF-α-induced activation of caspase-8, caspase-3 and apoptosis [22]. It has been reported that TNF-α induces phosphorylation of signaling pathways, such as PI3K/Akt. Thus, the JAK2/STAT3 pathway may play a major role in apoptosis in *Brucella*-infected cells via TNF-α-induction.

To further verify the above results, caspase-3, Bcl-2 and Bax were analyzed by RT-PCR or Western blot. Caspase-3 is one of the most important executors of apoptosis and is the main effector in the process of apoptosis [23]. Bcl-2 is an antiapoptotic gene belonging to the Bcl-2 family of proteins; Bax is a member of the Bcl-2 family, which can promote apoptosis [24]. The results of this study indicated that the expression of apoptosis-related proteins (caspase-3, Bax) were reduced in *Brucella* M5-90-infected RAW264.7 cells treated with AG490, however the results of Bc12 expression were opposite. Taken together, the results obtained in the current study demonstrate that the JAK2/STAT3 signaling pathway regulates apoptosis in M5-90-infected RAW264.7 cells at the transcriptional level and protein level.

Notably, Previous studies have indicated that the regulation of macrophage apoptosis is also one of the mechanisms by which *Brucella* escapes the immune system [25]. In this study, flow cytometry experiments showed that AG490 contributes to the inhibition of apoptosis, and the inhibition of the JAK2/STAT3 signaling pathway by AG490 decreased apoptotic rates in M5-90-infected RAW264.7 cells, other research show that *Brucella* infection-induced down-regulation of apoptotic level is an essential factor for the intracellular survival of *Brucella* within macrophages [25], this conclusion was also confirmed by this study. Thus, the JAK2/STAT3 signaling pathway indeed plays an important role in apoptosis of RAW264.7 cells infected with *Brucella* M5-90, thereby it is beneficial for survival and replication of *Brucella* M5-90. In addition, this study also found that secretion of TNF-α was positively related to transcriptional levels of caspase-3, Bax, and apoptosis rates. AG490 also affected the secretion of TNF-α. Thus, activation of the JAK2/STAT3 pathway could regulate *Brucella* M5-90 survival via induction of TNF-α.

In summary, the JAK2/STAT3 pathway is activated by *Brucella* M5-90 and plays an important role in regulating apoptosis of TNF-α-induced anti-*Brucella* activity. Inhibition of the JAK2/STAT3 pathway can inhibit Th1 immune responses, apoptosis rates, and intracellular survival of *Brucella*. This study may provide a preliminary theoretical basis for the study of the immune escape mechanism of

Brucella. However, the specific molecular mechanisms and the interactions between each signaling pathway needs to be examined in depth.

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