ABSTRACT

Background: Brucella infection induces brucellosis, a zoonotic disease. The intracellular circulation process and virulence of Brucella mainly depend on its type IV secretion system (T4SS) expressing secretory effectors. Secreted protein BspJ is a nucleomodulin of Brucella that invades the host cell nucleus. BspJ mediates host energy synthesis and apoptosis through interaction with proteins. However, the mechanism of BspJ as it affects the intracellular survival of Brucella remains to be clarified.

Objectives: To verify the functions of nucleomodulin BspJ in Brucella’s intracellular infection cycles.

Methods: Constructed Brucella abortus BspJ gene deletion strain (B. abortus ΔBspJ) and complement strain (B. abortus pBspJ) and studied their roles in the proliferation of Brucella both in vivo and in vitro.

Results: BspJ gene deletion reduced the survival and intracellular proliferation of Brucella at the replicating Brucella-containing vacuoles (rBCV) stage. Compared with the parent strain, the colonization ability of the bacteria in mice was significantly reduced, causing less inflammatory infiltration and pathological damage. We also found that the knockout of BspJ altered the secretion of cytokines (interleukin [IL]-6, IL-1β, IL-10, tumor necrosis factor-α, interferon-γ) in host cells and in mice to affect the intracellular survival of Brucella.

Conclusions: BspJ is extremely important for the circulatory proliferation of Brucella in the host, and it may be involved in a previously unknown mechanism of Brucella’s intracellular survival.

Keywords: Brucella abortus; secreted protein; intracellular survival; cytokines; clinical pathology

INTRODUCTION

Brucella infection can cause brucellosis of the host, a disease that manifests as miscarriage, infertility, and lameness in animals and fever and arthritis in humans [1]. Humans can be
Nucleomodulin BspJ promotes *Brucella*’s intracellular survival

Yuhe Miao
https://orcid.org/0000-0001-8271-591X
Yimei Xu
https://orcid.org/0000-0003-0013-4044
Ruirui Hu
https://orcid.org/0000-0002-0778-5941
Wei Zheng
https://orcid.org/0000-0003-3349-2104
Jiali Yi
https://orcid.org/0000-0001-4936-2385
Ruihu Li
https://orcid.org/0000-0001-1816-1693
Zhiquiang Li
https://orcid.org/0000-0002-1719-3502
Yong Wang
https://orcid.org/0000-0002-4263-8753
Chuangfu Chen
https://orcid.org/0000-0001-5381-6518

infected by inhaling aerosolized bacteria or by ingesting or contacting contaminated tissues or their derivatives. *Brucella melitensis*, *Brucella suis*, and *Brucella abortus* are highly pathogenic to humans. In addition, *Brucella canis* and *Brucella neotomae* can also cause infections in humans [2-5].

*Brucella* infection has consistent pathological and physiological characteristics at the animal and human cell levels [3,6]. After *Brucella* invades host cells, it hides in *Brucella*-containing vacuoles (BCV) [7] through endosomal BCV (eBCV), the replicating *Brucella*-containing vacuoles (rBCV), and autophagy BCV (aBCV) to complete the intracellular circulation process. Initially, the BCV travels along the endocytic pathway and is acidified after obtaining endosomal markers; at this point it is called the eBCV [8]. After the eBCV combines with the endocytic compartment, it loses endosomal markers and interacts with the endoplasmic reticulum (ER) to obtain ER membrane markers [9], forming an rBCV network that is conducive to the survival and replication of *Brucella* and thereby promoting the proliferation of the bacteria. Next, the rBCV undergoes recombination with vacuolar and autophagy properties (aBCV), triggering the release of *Brucella* [8,10].

The VirB type IV secretion system (T4SS) is an important regulatory system of *Brucella*. A number of studies have confirmed that the expression of T4SS is necessary for *Brucella* replication [10-13]. Similar to other intracellular parasites [14,15], *Brucella*’s VirB T4SS can transport effector proteins into host cells to regulate specific cellular functions. Studies have reported that the *Brucella* effector proteins not only play an important role in the rBCV stage but are also important in the aBCV stage [16]. In recent years, many *Brucella* secreted proteins have been discovered, including VceC [17,18], BtpA/Btp1/TcpB [19-25], BspA, BspB, and BspF [26,27]. The secreted protein BspJ (BAB_RS26920) is a newly discovered putative effector protein of *Brucella* [26]. Recently, we have identified BspJ as a nucleomodulin of *Brucella* and found that it invades the host cell nucleus, interacts with the host cell CKB and NME2 protein of *Brucella* and then is induced in the host cell nucleus, interacts with the host cell CKB and NME2 protein of *Brucella* and thereby promotes the proliferation of the bacteria. This may have a nuclear cell shuttle mechanism [28]. However, the mechanism of BspJ in the intracellular survival and circulating proliferation of *Brucella* has not yet been elucidated.

*B. abortus* was first found in the placenta of infected animals, and it has played an important role in the characteristics of *Brucella* infection and intracellular circulation [16,29]. We constructed a BspJ deletion mutant of *B. abortus* (*B. abortus ΔBspJ*) and a complement strain (*B. abortus pBspJ*) and verified the changes in their main biological characteristics and their functions of intracellular survival *Brucella in vivo* and *in vitro*. We identified an important role played by the nucleomodulin BspJ, and the results provide new insights into the pathogenic mechanism of *Brucella*.

**MATERIALS AND METHODS**

**Strains, cells, and animals**

*B. abortus* was provided by the China Center for Disease Control and Prevention (Beijing, China). *B. abortus* was cultivated with *Brucella* medium BBL *Brucella* Broth (BD, USA) or BBL *Brucella* Agar (BD, USA) at 37°C. All *Brucella*-related operations were performed in the BSL3 laboratory. *Escherichia coli* DH5α was obtained from the Collaborative Innovation Center for the Prevention and Control of Infectious Diseases of Western China and was cultured using LB (Luria-Bertani) medium. Mouse macrophages RAW264.7 were obtained from Procell (Wuhan, China) and were cultured in DMEM medium with 10% Fetal Bovine Serum (FBS)
Gibco, USA) under 5% CO₂. Total of 60 female 6-week-old BalB/c SPF mice were provided by SiPeiFu (Beijing, China). All animals met the standards of animal welfare and were treated humanely.

**Construction of *B. abortus* ΔBspJ, and *B. abortus* pBspJ**

The gene database (https://www.ncbi.nlm.nih.gov) was searched to obtain the gene sequence of BspJ (BAB_RS26920) and to design its upstream homology arm primers bspj-U-F: GGCAGGAGGTGAAGGATGAATT, bspj-U-R: TGACATTATCCAGGTGGCTGCACTACCGTGCTTTTCAGAG; and downstream homology arm primers bspj-D-F: TCTGGGGTTCGAAATGACCGTGCCGAGGGAAAGCGCCG, bspj-D-R: GAAGACGCTCCGTATTACCGCA. The upstream homology arms of BspJ were amplified with bspj-U-F and bspj-U-R via PCR with the following protocol: 95°C for 40 s, 60°C for 30 s, and 72°C for 50 s, with 25 cycles. The downstream homology arms of BspJ were amplified with bspj-D-F and bspj-D-R using the PCR protocol of 95°C for 40 s, 58°C for 30 s, and 72°C for 55 s, with 25 cycles. Meanwhile, the Kanamycin gene was amplified with bspj-U-R and bspj-D-F as primers under the PCR conditions 95°C for 40 s, 62°C for 30 s, and 72°C 65 s, with 25 cycles. The BspJ upstream homology arm gene, Kanamycin gene, and BspJ downstream homology arm gene were subjected to the first round of fusion PCR (95°C 30 s, 65°C 30 s, 72°C 60 s, 10 cycles). bspj-U-F and bspj-D-R were added for the second round of PCR (95°C 30 s, 60°C 30 s, 72°C 180 s, 30 cycles). The fragments of the second round of PCR were collected and constructed into a pMD19-T vector (TaKaRa, Japan) and electrotransformed (1800 V, 400, 25 μF) to *B. abortus*. After screening and PCR identification, a BspJ gene deletion strain (*B. abortus* ΔBspJ) was constructed. In addition, we used bspj-F: ATGAAGAGCCTGCAGTTCTCCAAG and bspj-R: CCTGTAGGCCCTAGGCACGG to amplify the BspJ gene (95°C 30 s, 65°C 30 s, 72°C 40 s, 30 cycles) and constructed the BspJ gene into the pBBR1MCS-4 vector (Miaolingbio, China). The pBBR1MCS-4-BspJ vector was electrotransformed (1800V, 400, 25 μF) to *B. abortus* ΔBspJ. After screening and PCR identification, the BspJ gene complement strain (*B. abortus* pBspJ) was obtained.

**Identification and growth characteristics analysis of *B. abortus* ΔBspJ, and *B. abortus* pBspJ**

We performed western blot analysis on the BspJ protein in *B. abortus, B. abortus* ΔBspJ and *B. abortus* pBspJ to identify the expression levels of BspJ in the parental strain, the deletion strain, and the complement strain. A total bacterial protein extraction kit (CWBiO, China) was used to extract the total bacterial protein, and then SDS-PAGE was performed to separate the proteins, after which the target proteins were transferred to a PVDF membrane under constant voltage. The PVDF membrane was blocked with 5% skim milk at 37°C for 2 h, washed with TBST, and incubated at 37°C with rabbit anti-BspJ and BspG protein polyclonal antibodies (1:200) (obtained from previous experiments, unpublished) for 2 h. Then, the membranes were incubated with goat anti rabbit IgG H&L (1:3,000) (Abcom, USA) antibody at 37°C for 1 h, and finally, Pierce ECL Western Blotting Substrate (Thermo, USA) was added for color development.

Single colonies of *B. abortus, B. abortus* ΔBspJ, and *B. abortus* pBspJ were selected and cultured with *Brucella* Broth medium at 37°C to an OD₆₀₀ values of 0.2. The cultures were then newly inoculated into *Brucella* Broth (1:100) at 37°C and 180 rpm to continue culturing. The OD₆₀₀ values of the bacterial solution were measured every 2 h, and the growth rate of the strains were recorded and used to draw a growth curve.
Analysis of adhesion and invasiveness of \textit{B. abortus} ΔBspJ, and \textit{B. abortus} pBspJ

Next, we studied the effect of BspJ gene deletion on the adhesion and invasiveness of \textit{B. abortus}. Cultured RAW264.7 cells on the order of $2 \times 10^6$ cells/well in six-well plates, and infected RAW264.7 cells with \textit{B. abortus}, \textit{B. abortus} ΔBspJ, and \textit{B. abortus} pBspJ according to MOI 100. At 15 min, 30 min, 45 min, and 60 min post-infection, gentamicin (50 $\mu$g/mL) were added to kill extracellular bacteria, and 0.3% Triton X-100 (Solarbio, China) were added 1 h later to lyse the cells. The cell lysates were diluted to $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ gradients to spread on \textit{Brucella} Agar plates and incubated at 37°C for 3–4 days, after which the numbers of colonies on the plates were counted.

\textit{In vitro} verification of the intracellular viability of \textit{B. abortus} ΔBspJ, and \textit{B. abortus} pBspJ

We subsequently studied the effect of BspJ gene knockout on the intracellular viability of \textit{B. abortus}. RAW264.7 cells were cultured in six-well plates with an order of magnitude of $2 \times 10^6$ cells/well. The cells were infected with \textit{B. abortus}, \textit{B. abortus} ΔBspJ or \textit{B. abortus} pBspJ at MOI 100. After 1 h of infection, gentamicin (50 $\mu$g/mL) were used to kill extracellular bacteria. At 4 h, 8 h, 12 h, 24 h, and 48 h after infection, 0.3% Triton X-100 (Solarbio, China) were added to lyse the cells to release intracellular bacteria. The lysates were diluted to $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$. The dilutions were spread on \textit{Brucella} Agar plates, and the numbers of colonies on the plates were counted after culturing at 37°C for 3–4 days.

Establishment of mouse infection models

All experimental procedures and animal care protocols were performed in accordance with institutional animal care regulations. Six-week-old female BalB/c mice were randomly divided into three groups, and injected intraperitoneally with \textit{B. abortus}, \textit{B. abortus} ΔBspJ, or \textit{B. abortus} pBspJ at a dose of $1 \times 10^6$ CFU/mouse. After infection, the mice were weighed every week. The mice were sacrificed using CO$_2$; the organs of the mice were collected aseptically, and the mouse serum was collected, observed, and recorded over a period of four weeks. The mouse organs were added to 0.25% Trixton-100 (Solarbio, China) to produce a homogenate, diluted to three gradients of $10^{-1}$, $10^{-2}$, and $10^{-3}$, spread on \textit{Brucella} Agar plates, and cultured at 37°C for 3–4 days to count the number of colonies on the plates.

Observation of pathological changes

Using 5% paraformaldehyde (Biosharp, China), the organs of different groups of mice collected at different time periods were permeabilized and fixed. After 15 days, organ samples were embedded in paraffin blocks, and the tissues were sectioned with a thickness of 4–6 $\mu$m and stained with hematoxylin-eosin (HE) for pathological sectioning. The pathological changes of the tissues were observed under a microscope, photographed, and recorded.

Analysis of cytokine changes \textit{in vivo} and \textit{in vitro}

Mouse macrophages RAW264.7 were infected with \textit{B. abortus}, \textit{B. abortus} ΔBspJ, or \textit{B. abortus} pBspJ, filtered, sterilized, and collected at 4 h, 8 h, 12 h, 24 h, and 48 h as cell supernatants. \textit{B. abortus}, \textit{B. abortus} ΔBspJ, and \textit{B. abortus} pBspJ were used to infect BalB/c mice, and serum was collected at 7 d, 14 d, 21 d, and 28 d. The enzyme-linked immunosorbent assay method was used to determine the cytokine expression levels of interleukin (IL)-6 (Mlbio, China), IL-10 (Mlbio, China), IL-1β (Mlbio, China), tumor necrosis factor-α (J&L Biological, China), and interferon-γ (J&L Biological, China) in cell supernatants or in mouse serum.
Data analysis
In our experimental study, each set of experimental data represents the average of repeated experiments at three levels. SPSS Statistics 23 was used to analyze correlations. One-way analysis of variance and Student’s t-test were used to test for significant differences between groups. The p value represents the degree of significance. The figures were composed using GraphPad Prism.

Ethics statement
The animal study was reviewed and approved by the Animal Experimental Ethical Inspection of First Affiliated Hospital, Shihezi University School of Medicine (Approval Number A2020-129-01).

RESULTS
Acquisition of B. abortus ΔBspJ, and B. abortus pBspJ
First, we measured the expression levels of BspJ protein in B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ strains; BspG protein was used as a control. The results of western blot analysis showed that the BspJ protein was successfully detected in B. abortus and B. abortus pBspJ. BspJ was not expressed in B. abortus ΔBspJ (Fig. 1A), while the BspG protein was normally expressed.

Fig. 1. Identification and growth characteristics analysis of B. abortus ΔBspJ. (A) Western blot analysis of BspJ protein in different strains. Equal amounts of B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ were collected. After extracting the total bacterial protein, SDS-PAGE was performed to separate proteins. The proteins were incubated with antibodies for the western blot. (B) Semi-quantitative analysis of BspJ expression. ImageJ software was used to analyze the expression levels of BspJ and express it in the form of a histogram. (C) Growth curves of B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ. After the strains were inoculated into the culture medium, the absorbance of the culture solution was measured every 2 h to evaluate the growth rate and number of strains. The graphs represent the results of three independent trials. All values were presented as means ± SD, and significant differences were represented by asterisks.

* p < 0.05, **** p < 0.0001.
in the three strains. We then performed a semi-quantitative analysis of the western blots and found that the expression of BspJ in B. abortus pBspJ was slightly higher than that of B. abortus (Fig. 1B), indicating that the backfilling of BspJ increased the expression of BspJ. These results indicate that B. abortus ΔBspJ and B. abortus pBspJ strains were successfully constructed.

**Knockout of BspJ reduced the growth rate of B. abortus**
We examined the effect of BspJ knockout on the proliferation of B. abortus. The growth rates of B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ were used to construct the respective growth curves. Compared with the parent strain, B. abortus ΔBspJ and B. abortus pBspJ always had lower growth rates, and the rate of B. abortus pBspJ was slightly higher than that of B. abortus ΔBspJ. In addition, B. abortus entered the exponential growth phase and plateau phase earlier than B. abortus ΔBspJ or B. abortus pBspJ and had a higher environmental capacity (Fig. 1C). These results confirmed that the lack of BspJ reduced the proliferation rate and viability of B. abortus and that the supplementation of BspJ did not completely restore bacterial viability, implying the important role of BspJ in the survival of B. abortus.

**Knockout of BspJ reduced the intracellular survival of B. abortus in RAW264.7**
Next, we examined the impact of BspJ knockdown on the intracellular survival of B. abortus in the host cells. The B. abortus parent strain, B. abortus ΔBspJ, and B. abortus pBspJ were used to infect RAW264.7 cells. Within 4–12 h after infection, compared with the parent strain and the complement strain, there was no visible difference in the cell number or intracellular survival of the BspJ gene-deficient strain, and the number of bacteria increased slowly in the host cell, with basically no increase within the first 12 h. However, after 24 h of infection, the bacteria proliferated in the host cells more rapidly. Compared with the parent strain, the number of intracellular bacteria of B. abortus ΔBspJ was decreased (p < 0.01), and the difference was more significant after 48 h (p < 0.001), while B. abortus pBspJ had no difference in intracellular survival compared with the parent strain (Fig. 2A). These results indicate that after Brucella invades the host cell, the growth of the bacteria is slow within the first 12 hours; the replication ability increases after 12 hours, and the deletion of the BspJ gene can inhibit the Brucella proliferation and reduce its intracellular viability in the cell to a certain extent.
Knockout of BspJ does not reduce the adhesion or invasion ability of *B. abortus*

By evaluating intracellular viability, we confirmed that the absence of BspJ would reduce the proliferation of bacteria. Subsequently, we assessed whether the knockout of BspJ affected the intracellular growth rate by reducing the adhesion and invasion efficiency of the bacteria. The results showed that within 1 h of the bacteria infecting the macrophage RAW264.7, the numbers of *B. abortus*, *B. abortus ΔBspJ*, and *B. abortus pBspJ* in cells were not significantly different, and with increasing time after invasion, the number of bacteria in the cells did not increase significantly (Fig. 2B). Compared with the parent strain, the adhesion and invasion abilities of *B. abortus ΔBspJ* in RAW264.7 cells were unaffected, and the number of adherent bacteria did not increase with time. This shows that a lack of BspJ does not reduce the ability of *B. abortus* to proliferate by affecting the adhesion or invasion of the bacteria.

**Absence of BspJ changes the expression of cytokines in mouse RAW264.7 cells**

Compared with the parent strain, *B. abortus ΔBspJ* had a reduced proliferation ability in RAW264.7 cells. To clarify the mechanism of this result, we analyzed the cytokines expression in the cell supernatants after the strains had infected the cells by examining the expression of TNF-α, IFN-γ and inflammatory cytokines IL-1β, IL-6, and IL-10 (Fig. 3). After *B. abortus*, *B. abortus ΔBspJ*, and *B. abortus pBspJ* infected RAW264.7 cells, within 8 h the IL-6 expression induced by *B. abortus ΔBspJ* was lower than that of the parent strain of *B. abortus* (Fig. 3B), while the expression levels of other cytokines showed no significant differences. With the increase of infection time, *B. abortus ΔBspJ* induced host cells to secrete IL-1β and IL-10 less than the parent strain, and the decrease of IL-10 (post-infection 12 h) occurred earlier than that of IL-1β (24 h post-infection) (Fig. 3A and C). It is worth noting that starting from 24 h after infection, *B. abortus ΔBspJ* induced higher levels of TNF-α and IFN-γ in host cells compared with the parent strain, and the difference in IFN-γ was more significant (Fig. 3D and E), consistent with the time when the proliferation rate of *B. abortus ΔBspJ* began to decrease (Fig. 2A). These results indicate that compared with the parent strain of *B. abortus*, *B. abortus ΔBspJ* reduces the expression of IL-6 in host cells at the initial stage of infection and reduces the expression of IL-10 in host cells 12 h after infection. During the bacterial proliferation stage, *B. abortus ΔBspJ* induced higher expression of TNF-α and IFN-γ in host cells. These results confirmed that the knockout of BspJ altered the expression of inflammatory and immune factors in host cells.

**The absence of BspJ reduced the colonization of *B. abortus* in mice and reduced pathological damage**

In order to observe the effect of BspJ deletion on the colonization of *B. abortus in vivo*, we infected BalB/c mice with the parental, deleted, and complement strains. After four consecutive weeks of follow-up observation, *B. abortus* caused more severe splenomegaly and damage in mice than *B. abortus ΔBspJ* (Fig. 4A), and this splenomegaly injury had first appeared seven days after infection (Fig. 4B). The spleen enlargement caused by *B. abortus* reached a peak on the 14th day and then began to slowly decrease (Fig. 4B), while the spleen swelling responses induced by *B. abortus ΔBspJ* and *B. abortus pBspJ* did not change much, maintaining a relatively stable level (Fig. 4B). In order to better understand the relationship between splenomegaly response and bacterial infection, we performed a statistical analysis on the colonization of *Brucella* in the spleens. After *Brucella* infected the mice, the bacterial content in the spleens reached its peak at 7–14 days and then began to slowly decrease (Fig. 4C), consistent with the response time axis of splenomegaly. From beginning to end, *B. abortus ΔBspJ* had a significantly lower spleen bacterial load than *B. abortus*, and the difference became greater as the infection time increased, following the same pattern as the
spleenomegaly. It is worth noting that \textit{B. abortus} pBspJ was higher than \textit{B. abortus} ΔBspJ but lower than \textit{B. abortus} in both splenomegaly reaction and splenic bacterial loads.

Next, we performed pathological observations on the mouse spleen and liver to understand the pathological damage caused by \textit{Brucella} to the mice. The results showed that 14 days after infection, \textit{B. abortus} caused more severe necrosis and inflammatory cell infiltration in the

![Fig. 3. Absence of BspJ altered the expression of cytokines in vitro. \textit{B. abortus}, \textit{B. abortus} ΔBspJ, and \textit{B. abortus} pBspJ infected RAW264.7 cells. The cell culture supernatants were collected at 4 h, 8 h, 12 h, 24 h, and 48 h after infection. The enzyme-linked immunosorbent assay was used to measure the expression levels of each cytokine in the supernatants. (A) The expression levels of IL-1β in the cell supernatants. (B) The expression levels of IL-6 in the cell supernatants. (C) The expression levels of IL-10 in the cell supernatants. (D) The expression levels of TNF-α in the cell supernatants. (E) The expression levels of IFN-γ in the cell supernatants. The graphs represent the results of three independent trials. All values were presented as means ± SD, and the significance of the difference was represented by asterisks. IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. * \(p < 0.05\), ** \(p < 0.01\).]
Nucleomodulin BspJ promotes Brucella’s intracellular survival

Liver of mice than B. abortus ΔBspJ or B. abortus pBspJ. At 28 days after infection, although the damage and infiltration caused by the strains were alleviated, the damage and lesions caused by B. abortus were still more intense than those from the deletion strain and the complement.

Fig. 4. The knockout of BspJ reduced the colonization of B. abortus in mice and reduced pathological damage. (A) B. abortus ΔBspJ infection caused mild splenomegaly. Scale bar, 0.5 cm. (B) The proportion of body weight of the spleen in mice after bacterial infection. B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ infected Balb/c mice. The spleens were taken every week for clinical observation of symptoms. Spleens were weighed and photographed and recorded for four weeks. (C) The counts of B. abortus ΔBspJ in the spleens of mice were significantly reduced. Spleens of mice infected with B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ were harvested; the bacterial loads in the spleens were assayed by counting the number of colonies. (D) B. abortus ΔBspJ induced milder pathological damage in mice. The livers and spleens of mice infected with B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ were harvested; the tissues were fixed, and hematoxylin-eosin sections were made to observe the pathological changes. Scale bar, 50 μm. (E) The scores of mouse liver lesions were counted and analyzed. (F) Statistics and analysis of scores of mouse spleen lesions. The graphs represent the results of three independent trials. All values were presented as means ± SD, and the significance of differences was represented by asterisks.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
strain (Fig. 4D). In the spleens of infected mice, at both 14 days and 28 days, *B. abortus* also caused stronger tissue damage and more lesions than *B. abortus ΔBspJ* or *B. abortus pBspJ*. Note that *B. abortus ΔBspJ* had the weakest effect on the liver and spleen of mice, and no specific lesions or tissue damage were observed (Fig. 4D). In order to better evaluate the pathological damage caused by different strains, we carried out statistical analyses of the organ pathological scores. *Brucella* infection caused spleen and liver damage to the mice. *B. abortus ΔBspJ* caused extremely less damage to the liver and spleen of mice than *B. abortus* or *B. abortus pBspJ*, and *B. abortus pBspJ* caused less damage than *B. abortus* (Fig. 4E and F). Based on the above results, the absence of BspJ caused low splenomegaly and infiltration, reduced the colonization of *B. abortus* in mice, and caused only minor pathological tissue damage, implying that BspJ plays an important role in the proliferation and disease mechanisms of *B. abortus*.

**Knockout of BspJ changed the secretion of cytokines in mice**

In order to better understand the mechanism of BspJ deletion causing *B. abortus* to weaken the pathogenicity to mice, we detected the secretion levels of the cytokines TNF-α and IFN-γ and the inflammatory factors IL-1β, IL-6, and IL-10 in the serum of mice. At 7 to 28 days after *B. abortus* and *B. abortus ΔBspJ* infection in the mice, there was little difference in the secretion of IL-1β in serum (Fig. 5A). From 14 days to 28 days after infection, *B. abortus ΔBspJ* induced lower IL-6 secretion in mouse serum than *B. abortus* (Fig. 5B). Starting from 21 days after infection, *B. abortus ΔBspJ* also induced a lower level of IL-10 secretion in mouse serum compared with *B. abortus* (Fig. 5C). In addition, *B. abortus ΔBspJ* induced a higher level of TNF-α secretion in mice than *B. abortus* (Fig. 5D). It is worth noting that from the beginning of infection, *B. abortus ΔBspJ* significantly increased the secretion of IFN-γ in mouse serum, and this difference became greater as the infection time increased (Fig. 5E). The above results indicate that *B. abortus ΔBspJ* induces lower levels of IL-6 and IL-10 and higher levels of TNF-α and IFN-γ secretion in mouse serum than *B. abortus*, while IL-10 secretion is not significantly different in the serum.

**DISCUSSION**

The secreted proteins of *Brucella* are closely related to its intracellular survival mechanism and afford the bacterium with antigenicity and immune protection [16,30,31]. To date, more than a dozen *Brucella* T4SS effector proteins have been identified as playing important roles in the production of rBCV and in the intracellular circulation of *Brucella* [16,32-34]. *Brucella* secreted protein VceC [17,18] targets the host cell Grp78/BiP, activates the unfolded protein response (UPR), and triggers an inflammatory response. The T4SS effector protein TcpB [25] can inhibit TLR (Toll/IL-1 receptor) signals and induce the UPR. The T4SS effector proteins BspA, BspB, and BspF can inhibit host cell secretion and promote the proliferation of *Brucella* in host cells [26,27]. Our previous research found that BspJ functions as a nucleomodulin, mediating host energy synthesis and cell apoptosis pathways. In order to better understand the functions of BspJ, we constructed the BspJ gene deletion strains and complement strains. Using both *in vivo* and *in vitro* experiments, we have demonstrated that the knockout of BspJ reduces the proliferation efficiency of *B. abortus*, significantly weakens its viability in the host cell, eliminates the pathological tissue damage to the host, and alters the release of host cytokines.

*Brucella* in the early stage of invading host cells (0–8 h after infection) manifests as the eBCV stage. This stage is part of the endosomal stage and does not have the ability to proliferate bacteria. At the rBCV stage (12 to 48 hours after infection), *Brucella* will rapidly proliferate in host cells in large quantities [16,33,34]. In our research results, neither the *B. abortus* parent...
strains nor the B. abortus ΔBspJ and B. abortus pBspJ strains were observed at the initial stage of infection (0–12 h after infection). The proliferation of Brucella was remarkable. In the rBCV stage, rapid replication of Brucella in the cell was observed, and the proliferation of B. abortus ΔBspJ was significantly weaker than that of the B. abortus parent strain.

After Brucella infects macrophages, the host cells secrete some pro-inflammatory cellular immune factors (TNF-α, IL-6, IL-12) and chemokines [35,36] that in turn stimulate Th1 immune response. Nucleomodulin BspJ promotes Brucella’s intracellular survival.

Fig. 5. The deletion of BspJ altered the expression of cytokines in vivo. B. abortus, B. abortus ΔBspJ, and B. abortus pBspJ infected Balb/c mice. Sera of mice were collected weekly for enzyme-linked immunosorbent assay detection, and the expression levels of each cytokine in the mouse serum were calculated. (A) The expression levels of IL-1β in mouse serum. (B) IL-6 expression in mouse serum. (C) IL-10 expression in mouse serum. (D) The expression levels of TNF-α in mouse serum. (E) Expression of IFN-γ in mouse serum. The results were from three independent experiments. All values were presented as means ± SD, and the significance of differences was represented by asterisks. IL, interleukin; TNF, tumor necrosis factor; IFN, interferon. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. ns, no significant difference.
cell responses. IFN-γ, which is extremely important for the elimination of Brucella [37], is produced by the host cells. After LPS stimulates the host cell to activate the NF-κB signaling pathway, it will cause high expression of innate immune cytokines such as IL-6, IL-8, IL-1β, and TNF-α, and the activation of the STAT3 signaling pathway will up-regulate the production of IL-10 [38-40]. IL-10 has anti-inflammatory effects. Its secretion can reduce the expression of pro-inflammatory cytokines (such as TNF-α and IFN-γ), inhibit the Th1 cell response, regulate macrophage metabolism, and reduce the production of reactive oxygen species (ROS) and activate the inflammasomes [41,42]. In our research B. abortus ΔBspJ was used to infect mouse macrophages RAW264.7. Compared with the parental strain, IL-6 expression was reduced at the eBCV stage, and at the rBCV stage, IL-1β and IL-10 levels were reduced. The expression levels of TNF-α and IFN-γ were increased. This shows that during the proliferation stage of Brucella, the deletion of BspJ down-regulates the expression of IL-10 and enhances the inflammatory response. As a result, the expression levels of inflammatory killer cytokines TNF-α and IFN-γ increase, causing host cells to destroy the intracellular bacteria, resulting in a decrease in the proliferation rate of B. abortus ΔBspJ and a decrease in bacterial intracellular survival. In addition, the inflammatory cytokines IL-6 and IL-10 may not play significant roles in the survival of B. abortus ΔBspJ in cells. These suggestions need to be further verified.

For the in vivo experiments in mice, B. abortus ΔBspJ invasion reduced the inflammatory infiltration of mouse organs from the early stage; the colonization ability of the bacteria in the mouse spleen was significantly reduced, and the damage to the mouse organs was also significantly lessened. TcpS is an effector protein of Salmonella. Its deletion induces a decrease in the colonization of the host and early spleen and a strong inflammatory storm, indicating its important role in the early immune escape of Salmonella [39]. Our results show that the function of BspJ after entering the nucleus may not be related to the immune escape of the bacteria that enhances its cell memory viability. It is worth noting that in our results, the deletion of BspJ decreased the expression levels of IL-6 and IL-10 in the serum of mice at the later stages of infection, while the expression levels of TNF-α and IFN-γ were increased. This is consistent with our results in macrophages, indicating that the absence of BspJ may reduce the expression level of IL-10 to enhance the host cell inflammatory response and to cause the cell to secrete more cytokines (TNF-α and IFN-γ) to eliminate pathogenic bacteria. However, B. abortus ΔBspJ showed low host memory viability at the early stage. Due to its ability to enter the host cell nucleus, this may be another undiscovered mechanism for the intracellular survival of Brucella. The research on the effector protein BspJ still has a long way to go. We may conclude that BspJ is an important effector protein of B. abortus, and its deletion causes a series of changes that ultimately affect the colonization ability of B. abortus. The important functions of BspJ need to be further explored. How BspJ functions as a nuclear effector protein, how it affects the proliferation ability of pathogenic bacteria, and whether its deletion mutants can be used as candidate vaccine strains will be the directions of our future research.

CONCLUSION

In conclusion, by constructing B. abortus ΔBspJ and B. abortus pBspJ and verifying their biological characteristics, we found that BspJ plays an important role in the proliferation of the Brucella rBCV stage. In view of BspJ being a nucleomodulin, it may possibly participate in Brucella to activate the body’s adaptive immune process to affect the expression of specific cytokines and ultimately maintain the intracellular circulation of the bacteria.
REFERENCES

1. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Front Microbiol. 2014;5:213.
PUBMED | CROSSREF

2. Goonaratna C. Brucellosis in humans and animals. Ceylon Med J. 2009;52(2):66.
CROSSREF

3. Atluri VL, Xavier MN, de Jong MF, den Hartigh AB, Tsolis RM. Interactions of the human pathogenic Brucella species with their hosts. Annu Rev Microbiol. 2011;65(1):523-541.
PUBMED | CROSSREF

4. Martirosyan A, Moreno E, Gorvel JP. An evolutionary strategy for a stealthy intracellular Brucella pathogen. Immunol Rev. 2011;240(1):211-234.
PUBMED | CROSSREF

5. Godfroid J, Cloeckaert A, Liautard JP, Kohler S, Fretin D, Walravens K, et al. From the discovery of the Malta fever’s agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res. 2005;36(3):313-326.
PUBMED | CROSSREF

6. Celli J. The changing nature of the Brucella-containing vacuole. Cell Microbiol. 2015;17(7):951-958.
PUBMED | CROSSREF

7. Comerci DJ, Martinez-Lorenzo MJ, Sieira R, Gorvel JP, Ugalde RA. Essential role of the VirB machinery in the maturation of the Brucella abortus-containing vacuole. Cell Microbiol. 2001;3(3):159-168.
PUBMED | CROSSREF

8. Starr T, Child R, Wehrly TD, Hansen B, Hwang S, López-Otin C, et al. Selective subversion of autophagy complexes facilitates completion of the Brucella intracellular cycle. Cell Host Microbe. 2012;11(1):33-45.
PUBMED | CROSSREF

9. Celli J, de Chastellier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel JP. Brucella evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med. 2003;198(4):545-556.
PUBMED | CROSSREF

10. Luizet JB, Raymond J, Lacerda TLS, Barbieux E, Kambarev S, Bonici M, et al. The Brucella effector BspL targets the ER-associated degradation (ERAD) pathway and delays bacterial egress from infected cells. Proc Natl Acad Sci U S A. 2021;118(32):e2105324118.
PUBMED | CROSSREF

11. Sieira R, Comerci DJ, Sánchez DO, Ugalde RA. A homologue of an operon required for DNA transfer in Agrobacterium is required in Brucella abortus for virulence and intracellular multiplication. J Bacteriol. 2000;182(17):4849-4855.
PUBMED | CROSSREF

12. Delrue RM, Martinez-Lorenzo M, Lestrange P, Danese I, Bielarz V, Mertens P, et al. Identification of Brucella spp. genes involved in intracellular trafficking. Cell Microbiol. 2001;3(7):487-497.
PUBMED | CROSSREF

13. O’Callaghan D, Cazevieille C, Allardet-Servent A, Boschiroli ML, Bourg G, Foulongne V, et al. A homologue of the Agrobacterium tumefaciens VirB and Bordetella pertussis Ptl type IV secretion systems is essential for intracellular survival of Brucella suis. Mol Microbiol. 1999;33(6):1210-1220.
PUBMED | CROSSREF

14. Green ER, Mecsas J. Bacterial secretion systems—an overview. Microbiol Spectr. 2016;4(1):4.
PUBMED | CROSSREF

15. Juhas M, Crook DW, Hood DW. Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell Microbiol. 2008;10(12):2377-2386.
PUBMED | CROSSREF

16. Celli J. The intracellular life cycle of Brucella spp. Microbiol Spectr. 2019;7(2):7.
PUBMED | CROSSREF

17. de Jong MF, Sun VH, den Hartigh AB, van Dijl JM, Tsolis RM. Identification of VceA and VoeC, two members of the VjbR regulon that are translocated into macrophages by the Brucella type IV secretion system. Mol Microbiol. 2008;70(6):1378-1396.
PUBMED | CROSSREF

18. de Jong MF, Starr T, Winter MG, den Hartigh AB, Child R, Knodler LA, et al. Sensing of bacterial type IV secretion via the unfolded protein response. MBio. 2013;4(1):e00418-e12.
PUBMED | CROSSREF

https://doi.org/10.4142/jvs.21224
19. Cirl C, Wieser A, Yadav M, Duerr S, Schubert S, Fischer H, et al. Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. Nat Med. 2008;14(4):399-406.

20. Radhakrishnan GK, Yu Q, Harms JS, Splitter GA. Brucella TIR domain-containing protein mimics properties of the Toll-like receptor adaptor protein TIRAP. J Biol Chem. 2009;284(15):9892-9898.

21. Sengupta D, Koblansky A, Gaines J, Brown T, West AP, Zhang D, et al. Subversion of innate immune responses by Brucella through the targeted degradation of the TLR signaling adapter, MAL. J Immunol. 2010;184(2):956-964.

22. Chaudhary A, Ganguly K, Cabantous S, Waldo GS, Micheva-Viteva SN, Nag K, et al. The Brucella TIR-like protein TcpB interacts with the death domain of MyD88. Biochem Biophys Res Commun. 2012;417(1):299-304.

23. Alaidarous M, Ve T, Casey LW, Valkov E, Ericsson DJ, Ullah MO, et al. Mechanism of bacterial interference with TLR4 signaling by Brucella Toll/interleukin-1 receptor domain-containing protein TcpB. J Biol Chem. 2014;289(2):654-668.

24. Jakka P, Namani S, Murugan S, Rai N, Radhakrishnan G. The Brucella effector protein TcpB induces degradation of inflammatory caspases and thereby subverts non-canonical inflammasome activation in macrophages. J Biol Chem. 2017;292(50):20613-20627.

25. Salcedo SP, Marchesini MI, Lelouard H, Fugier E, Jolly G, Balor S, et al. Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS Pathog. 2008;4(2):e21.

26. Myeni S, Child R, Ng TW, Kupko JJ 3rd, Wehrly TD, Porcella SF, et al. Brucella modulates secretory trafficking via multiple type IV secretion effector proteins. PLoS Pathog. 2013;9(9):e1003556.

27. Miller CN, Smith EP, Cundiff JA, Knodler LA, Blackburn JB, Lupashin V, et al. A Brucella type IV effector targets the COG tethering complex to remodel host secretory traffic and promote intracellular replication. Cell Host Microbe. 2017;22(3):317-329.e7.

28. Ma Z, Li R, Hu R, Deng X, Xu Y, Zheng W, et al. Brucella abortus BspJ is a nucleomodulin that inhibits macrophage apoptosis and promotes intracellular survival of Brucella. Front Microbiol. 2020;11:599205.

29. Dorneles EM, Teixeira-Carvalho A, Araujo MS, Sriranganathan N, Lage AP. Immune response triggered by Brucella abortus following infection or vaccination. Vaccine. 2015;33(31):3659-3666.

30. Liu Q, Liu Q, Yi J, Liang K, Liu T, Roland KL, et al. Outer membrane vesicles derived from Salmonella Typhimurium mutants with truncated LPS induce cross-protective immune responses against infection of Salmonella enterica serovars in the mouse model. Int J Med Microbiol. 2016;306(8):697-706.

31. Hayek I, Berens C, Lührmann A. Modulation of host cell metabolism by T4SS-encoding intracellular pathogens. Curr Opin Microbiol. 2019;47:59-65.

32. Ke Y, Wang Y, Li W, Chen Z. Type IV secretion system of Brucella spp. and its effectors. Front Cell Infect Microbiol. 2015;5:72.

33. Smith E, Cotto-Rosario A, Borghesan E, Held K, Miller C, Celli J. Epistatic interplay between type IV secretion effectors engages the small GTPase Rab2 in the Brucella intracellular cycle. MBio. 2020;11(2):e03350-19.

34. Borghesan E, Smith EP, Myeni S, Binder K, Knodler LA, Celli J. A Brucella effector modulates the Arf6-Rab8a GTPase cascade to promote intravacuolar replication. EMBO J. 2021;40(19):e107664.

35. Rittig MG, Kaufmann A, Robins A, Shaw B, Sprenger H, Gemsa D, et al. Smooth and rough lipopolysaccharide phenotypes of Brucella induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J Leukoc Biol. 2003;74(6):1045-1055.
36. Zaitseva M, King LR, Manischewitz J, Dougan M, Stevan L, Golding H, et al. Human peripheral blood T cells, monocytes, and macrophages secrete macrophage inflammatory proteins 1alpha and 1beta following stimulation with heat-inactivated *Brucella abortus*. Infect Immun. 2001;69(6):3817-3826.

37. Skendros P, Pappas G, Boura P. Cell-mediated immunity in human brucellosis. Microbes Infect. 2011;13(2):134-142.

38. Jaslow SL, Gibbs KD, Fricke WF, Wang L, Pittman KJ, Mammel MK, et al. *Salmonella* activation of STAT3 signaling by SarA effector promotes intracellular replication and production of IL-10. Cell Rep. 2018;23(12):3525-3536.

39. Xiong D, Song L, Geng S, Jiao Y, Zhou X, Song H, et al. *Salmonella* coiled-coil-and TIR-containing TcpS evades the innate immune system and subdues inflammation. Cell Rep. 2018;23(12):3525-3536.

40. Zheng M, Ambesi A, McKeown-Longo PJ. Role of TLR4 receptor complex in the regulation of the innate immune response by fibronectin. Cells. 2020;9(1):9.

41. Cyktor JC, Turner J. Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. Infect Immun. 2011;79(8):2964-2973.

42. Ip WK, Hoshi N, Shouval DS, Snapper S, Medzhitov R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science. 2017;356(6337):513-519.