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Nucleomodulin BspJ as an effector promotes the colonization of *Brucella abortus* in the host

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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

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Conflict of Interest

The authors declare no conflicts of interest.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors.

Author Contributions

Conceptualization: Ma Z, Wang Y, Chen C; Data curation: Ma Z; Formal analysis: Ma Z, Zhang H; Funding acquisition: Wang Y, Li Z, and Chen C; Investigation: Zheng W, Yi J; Methodology: Yu S, Cheng K; Project administration: Wang Y, Chen C; Resources: Miao Y, Xu Y; Software: Ma Z, Li R; Supervision: Wang Y, Chen C; Validation: Ma Z, Hu R; Visualization: Ma Z, Hu R; Writing - original draft: Ma Z, Wang Y; Writing - review & editing: Wang Y, Chen C.

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 proteins, mediates the host energy synthesis, metabolism, and apoptosis signaling pathways, and 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 studying 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% CO_2. 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: TGACATTCATCCCAGGTGGCTGCATCACCGTGCTTTCAGAG; 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 OD_{600} 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 OD_{600} 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 *B. abortus* ΔBspJ, and *B. abortus* pBspJ

Next, we studied the effect of BspJ gene deletion on the adhesion and invasiveness of *B. abortus*. Cultured RAW264.7 cells on the order of 2×10^6 cells/well in six-well plates, and infected RAW264.7 cells with *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ according to MOI 100. At 15 min, 30 min, 45 min, and 60 min post-infection, gentamicin (50 μg/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 *Brucella* Agar plates and incubated at 37°C for 3–4 days, after which the numbers of colonies on the plates were counted.

In vitro verification of the intracellular viability of *B. abortus* ΔBspJ, and *B. abortus* pBspJ

We subsequently studied the effect of BspJ gene knockout on the intracellular viability of *B. abortus*. RAW264.7 cells were cultured in six-well plates with an order of magnitude of 2×10^6 cells/well. The cells were infected with *B. abortus*, *B. abortus* ΔBspJ or *B. abortus* pBspJ at MOI 100. After 1 h of infection, gentamicin (50 μg/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 *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 *B. abortus*, *B. abortus* ΔBspJ, or *B. abortus* pBspJ at a dose of 1×10^6 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 *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 μm 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 *in vivo* and *in vitro*

Mouse macrophages RAW264.7 were infected with *B. abortus*, *B. abortus* ΔBspJ, or *B. abortus* pBspJ, filtered, sterilized, and collected at 4 h, 8 h, 12 h, 24 h, and 48 h as cell supernatants. *B. abortus*, *B. abortus* ΔBspJ, and *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

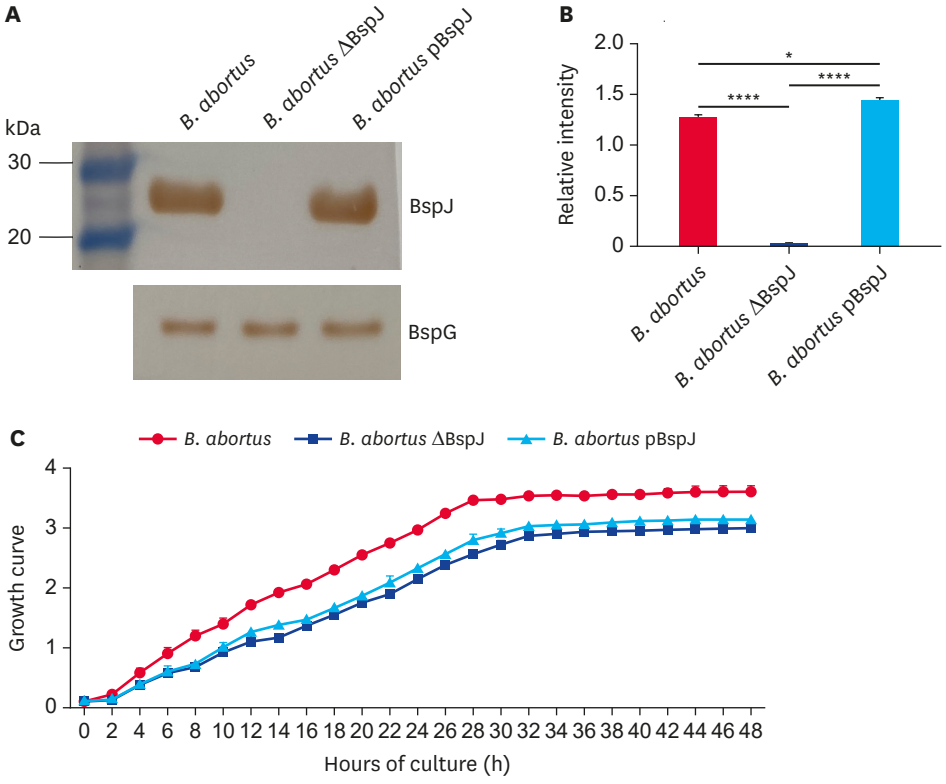

Fig. 1. Identification and growth characteristics analysis of *B. abortus* ΔBspJ. (A) Western blot analysis of BspJ protein in different strains. Equal amounts of *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ were collected. After extracting the total bacterial protein, SDS-PAGE was performed 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.
$^{*}p < 0.05$, $^{****}p < 0.0001$.

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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.

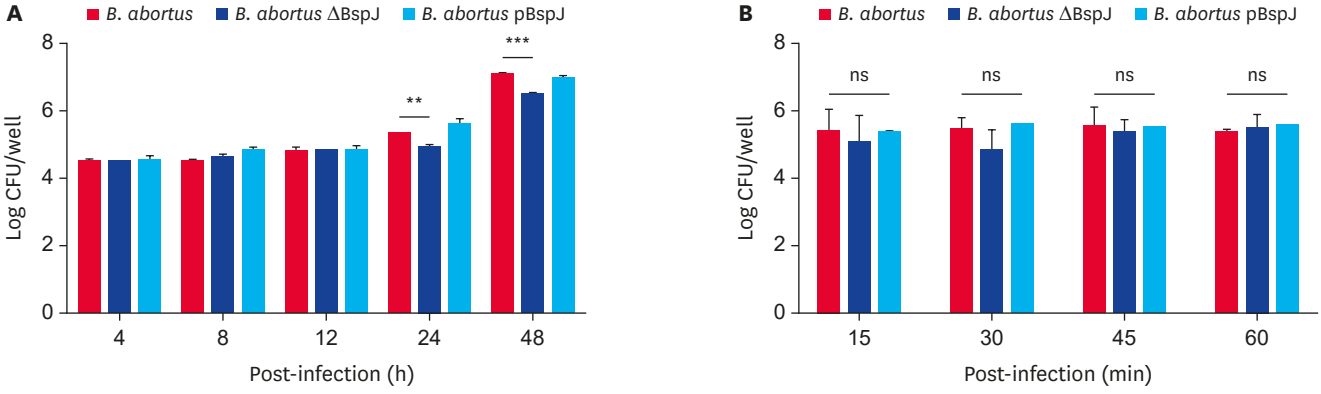

Fig. 2. Knockout of BspJ reduced the intracellular survival of *B. abortus*. (A) The intracellular survival of *B. abortus* decreased after BspJ deletion. *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ infected RAW264.7 cells. Collected 4 h, 8 h, 12 h, 24 h, and 48 h cell sample lysates to determine the intracellular survival of *Brucella*. (B) Knockout of BspJ did not reduce the adhesion and invasion ability of *B. abortus*. *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ infected macrophages RAW264.7. At 15 min, 30 min, 45 min, and 60 min, lysis solution was added to release *Brucella*, and the intracellular survival of *Brucella* was measured. The graphs represent the results of three independent experiments. All values were presented as means ± SD, and significant differences were represented by asterisks.
ns, no significant difference; $^{**}p < 0.01$, $^{***}p < 0.001$.

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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 results, 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-1β and 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

splenomegaly. It is worth noting that *B. abortus* pBspJ was higher than *B. abortus* ΔBspJ but lower than *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 *Brucella* to the mice. The results showed that 14 days after infection, *B. abortus* caused more severe necrosis and inflammatory cell infiltration in the

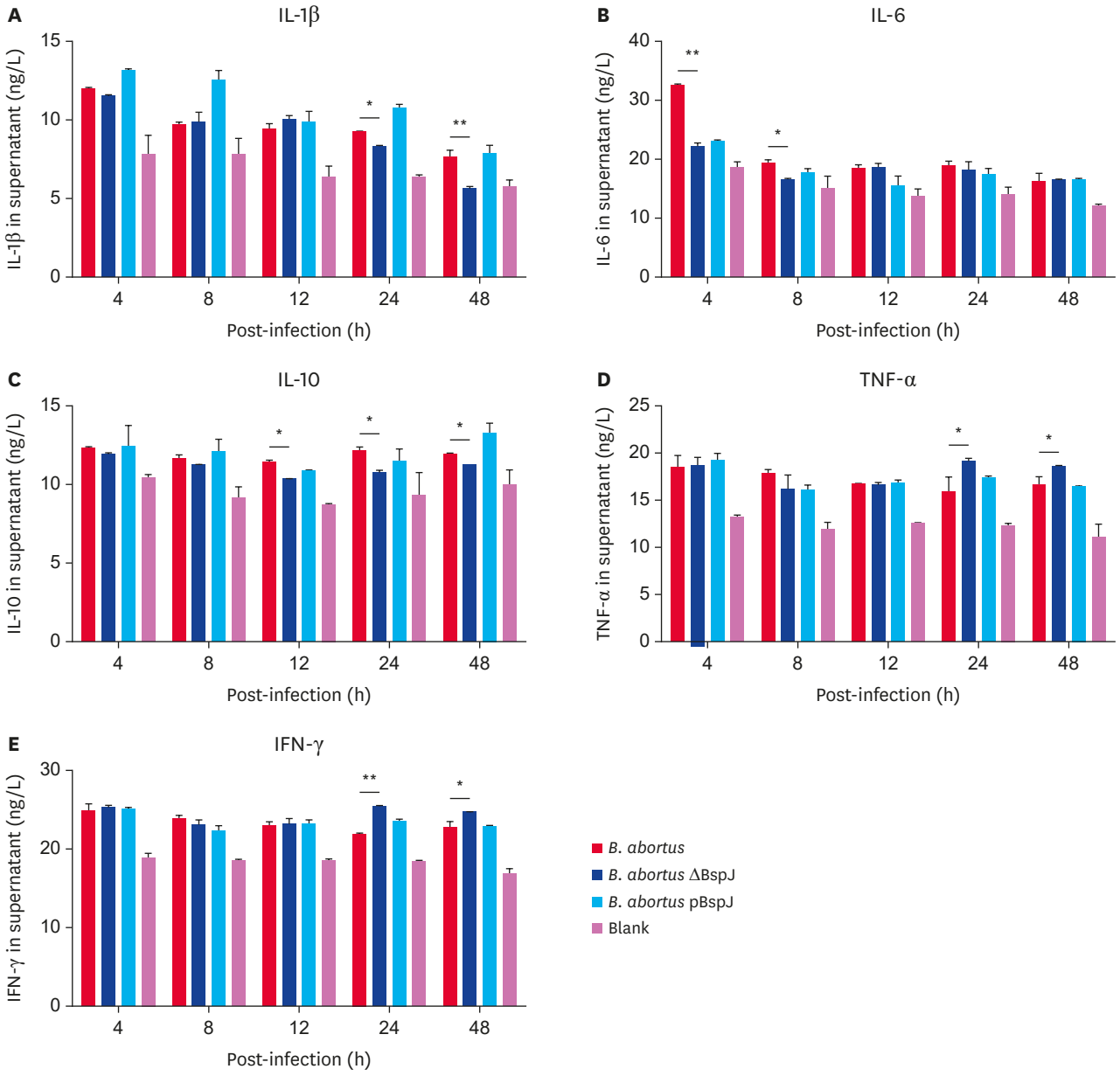

Fig. 3. Absence of BspJ altered the expression of cytokines *in vitro*. *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ infected RAW264.7 cells. The cell culture supernatants were collected at 4 h, 8 h, 12 h, 24 h, and 48 h after infection. The enzyme-linked immunosorbent assay was used to measure 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.
$^{*}p < 0.05$, $^{**}p < 0.01$.

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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

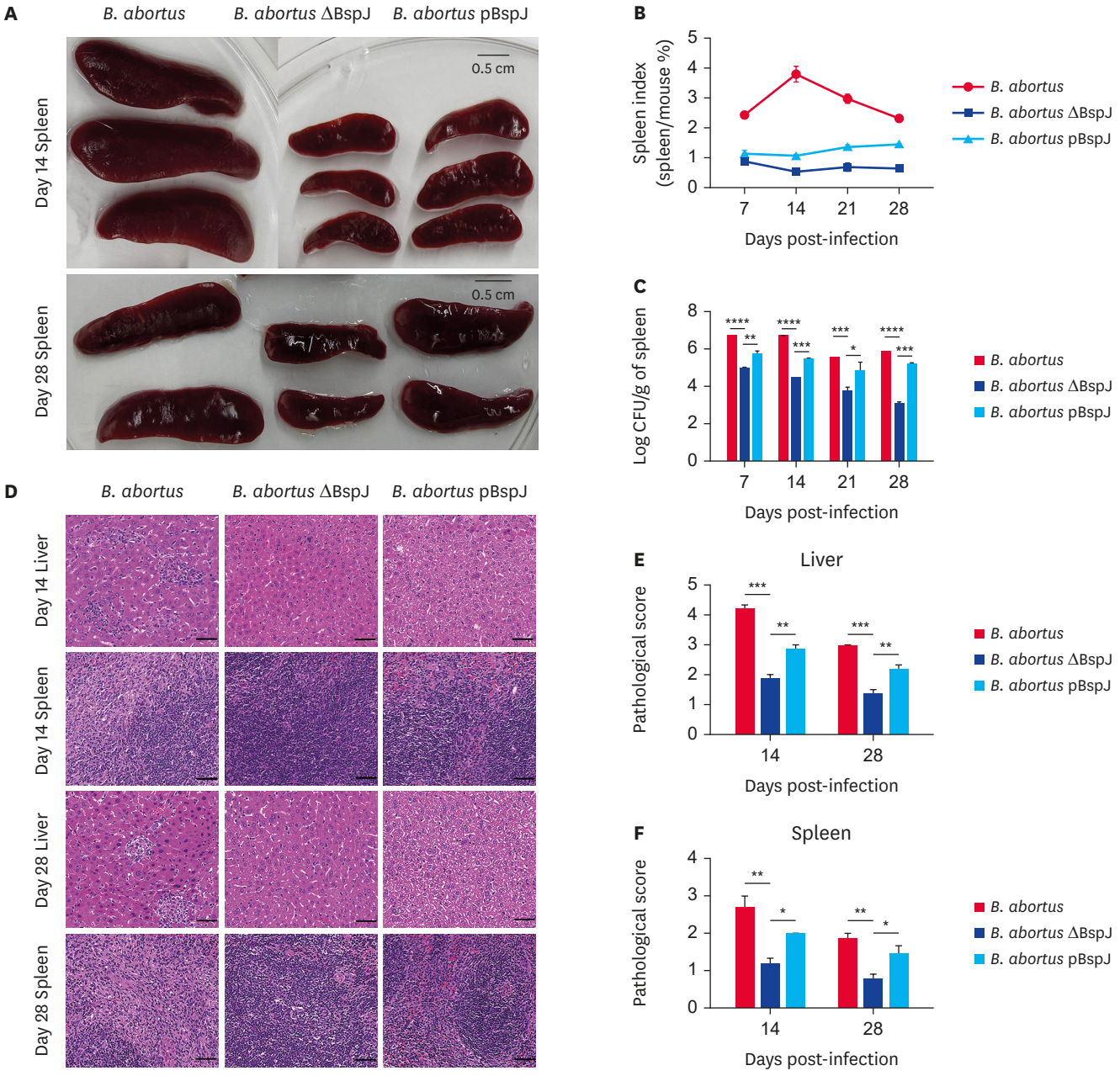

Fig. 4. The knockout of BspJ reduced the colonization of *B. abortus* in mice and reduced pathological damage. (A) *B. abortus* ΔBspJ infection caused mild splenomegaly. Scale bar, 0.5 cm. (B) The proportion of body weight of the spleen in mice after bacterial infection. *B. abortus*, *B. abortus* ΔBspJ, 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.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

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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

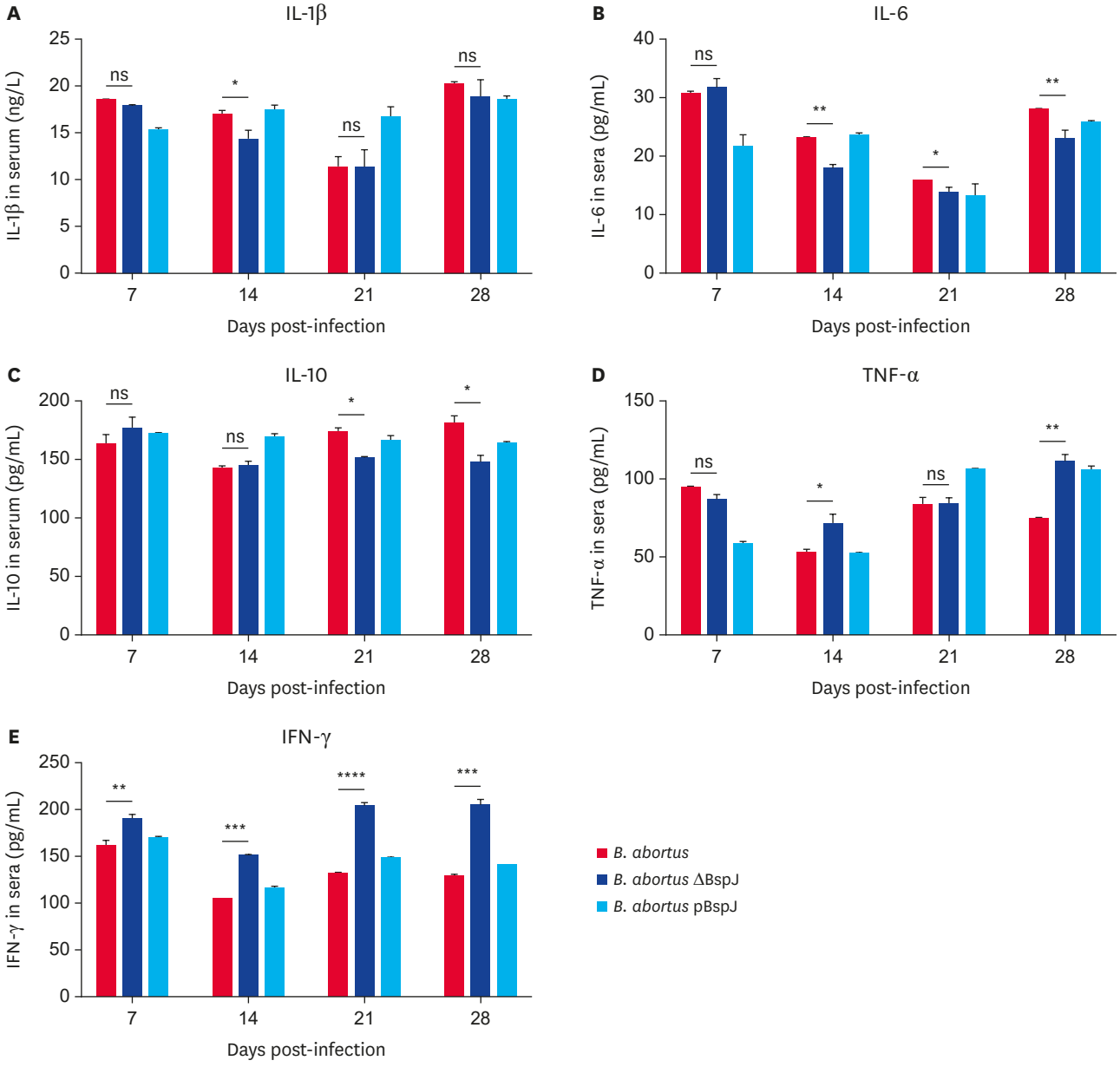

Fig. 5. The deletion of BspJ altered the expression of cytokines *in vivo*. *B. abortus*, *B. abortus* ΔBspJ, and *B. abortus* pBspJ infected BalB/c mice. Sera of mice were collected weekly for The enzyme-linked immunosorbent assay detection, and the expression levels of each cytokine in the mouse serum 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.
ns, no significant difference; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

strain 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

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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-1β 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.

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