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Interaction between *Brucella melitensis* 16M and small ubiquitin-related modifier 1 and E2 conjugating enzyme 9 in mouse RAW264.7 macrophages

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Conflict of Interest
The authors declare no conflicts of interest.

ABSTRACT

Brucella is an intracellular pathogen that invades a host and settles in its immune cells; however, the mechanism of its intracellular survival is unclear. Modification of small ubiquitin-related modifier (SUMO) occurs in many cellular activities. E2 conjugating enzyme 9 (Ubc9) is the only reported ubiquitin-conjugating enzyme that links the SUMO molecule with a target protein. *Brucella*'s intracellular survival mechanism has not been studied with respect to SUMO-related proteins and Ubc9. Therefore, to investigate the relationship between *Brucella melitensis* 16M and SUMO, we constructed plasmids and cells lines suitable for overexpression and knockdown of SUMO1 and Ubc9 genes. *Brucella* 16M activated SUMO1/Ubc9 expression in a time-dependent manner, and *Brucella* 16M intracellular survival was inhibited by SUMO1/Ubc9 overexpression and promoted by SUMO1/Ubc9 depletion. In macrophages, *Brucella* 16M-dependent apoptosis and immune factors were induced by SUMO1/Ubc9 overexpression and restricted by SUMO1/Ubc9 depletion. We noted no effect on the expressions of SUMO1 and Ubc9 in *B. melitensis* 16M lipopolysaccharide-prestimulated mouse RAW264.7 macrophages. Additionally, intracellular survival of the 16MΔVirB2 mutant was lower than that of *Brucella* 16M ($p < 0.05$). VirB2 can affect expression levels of Ubc9, thereby increasing intracellular survival of *Brucella* in macrophages at the late stage of infection. Collectively, our results demonstrate that *B. melitensis* 16M may use the VirB IV secretion system of *Brucella* to interact with SUMO-related proteins during infection of host cells, which interferes with SUMO function and promotes pathogen survival in host cells.

Keywords: *Brucella*; coupling enzyme Ubc9; interaction; intracellular; SUMO

INTRODUCTION

Brucella is a gram-negative intracellular bacterial pathogen whose infection can cause brucellosis in humans and other animals [1,2]. *Brucella* can invade blood and lymphatic vessels through wounds, and bacteria that escape being killed by the host's immune system eventually settle in macrophage cells and propagate, leading to persistent infection [3]. *Brucella* can also inhibit macrophage apoptosis, thereby evading the host's immune system.

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Author Contributions

Conceptualization: Wang Y; Data curation: Yi J, Wang Y; Formal analysis: Wang Y; Funding acquisition: Chen C; Investigation: Yi J, Li Q; Methodology: Wang Y, Deng X; Project administration: Chen C; Resources: Chen C; Software: Shao Z, Zhang H, and He J; Supervision: Wang Y, Xiao C; Validation: Chen C; Visualization: Wang Z; Writing - original draft: Yi J; Writing - review & editing: Chen C, Wang Y.

The VirB operon of *Brucella*, which encodes the type IV secretion system (T4SS), is required for its intracellular survival and persistence in the host [4]. However, how *Brucella* escapes the host's immune defenses, changes the intracellular environment, and invades host cells is incompletely described.

Small ubiquitin-related modifier (SUMO) modification, or SUMOylation, is a type of post-translational protein modification that greatly influences cellular processes [5], including signal transduction, immune recognition, apoptosis, inflammation, and antigen presentation [6,7]. The SUMO protein family has three isoforms, SUMO1, SUMO2, and SUMO3 that are associated with the following 4 ligase types, E1 activating enzyme, E2 activating enzyme, E2 conjugating enzyme 9 (Ubc9), and E3 ligase, all of which have major roles in protein modification [7,8]. SUMO1 has an important role in clearing bacteria via the immune system [9].

Brucella infection causes autophagy, inflammation reactions, and apoptosis of the host's macrophages, indicating that the mechanism of *Brucella* infection must be very complex [10]. However, whether the mechanism involves regulation of macrophages by SUMO or Ubc9 is unknown. Herein, we constructed the following cell lines: 1) a SUMO1 overexpression (O-S) cell line, 2) a Ubc9 overexpression (O-U) cell line, 3) a SUMO1 knockdown (K-S) cell line, and 4) a Ubc9 knockdown (K-U) cell line with which to explore the effects of SUMO1 and Ubc9 expression on inflammation, apoptosis, and the intracellular survival of *Brucella melitensis* 16M. We also explored the interaction between the T4SS-encoding VirB2 operon of *B. melitensis* 16M and the SUMO1/Ubc9 proteins in *Brucella*-infected cells. Our findings provide foundations for future studies on bacterial invasion and immune escape, chronic *Brucella* infection, and development of vaccines and drugs against persistent *Brucella* infection.

MATERIALS AND METHODS

Growth conditions for bacterial strains and cells lines

B. melitensis 16M (Chinese Academy of Agricultural Sciences, China) and the *B. melitensis* 16MΔ*VirB2* mutant strain (Key Laboratory of Zoonosis, Shihezi University, China) were grown on tryptic soy agar (Biosciences, USA). RAW264.7 macrophage cells and HEK-293FT cells were obtained from the Cell Resource Center, IBMS, CAMS/PUMC (China) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Plasmids and protein

The pLEX-green fluorescent protein (GFP) lentiviral overexpression vector, and the pSPAX2 and pMD2.G packaging vectors, were purchased from the Xinjiang Key Laboratory of Animal Epidemic Prevention and Control (China). Lentiviral RNA interference (RNAi) vector, pLL3.7-GFP, and the packaging plasmids RSV-REV and PCMV-VSVG were purchased from Xinjiang Key Laboratory of Animal Epidemic Prevention and Control (China). The pMD19-T cloning vector was purchased from Takara (China). VirB2 protein was obtained from The Key Laboratory of Zoonosis, Shihezi University (China).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays

RAW264.7 cells were cultured in 96-well culture plates (10^4 cells/well). The knockdown (pLL3.7-SUMO-1 and pLL3.7-Ubc9) and overexpressing (pLEX-SUMO-1 and pLEX-Ubc9) plasmids were transfected into RAW264.7 cells. Dimethyl sulfoxide (DMSO; 0.1%) was used

as a control. After treatment for 12, 24, 48, or 72 h, MTT (5 mg/mL) was added and the cells incubated for 4 h at 37°C. The supernatants were then removed and 150 μL DMSO was added to each well and the plate incubated at 37°C for 15 min. Absorbance at OD_{570} was measured for each well.

Construction of SUMO1 and K-U and overexpression vectors

We used the GenBank SUMO1 (NM009460.2) and Ubc9 (NM_001177610) gene sequences to design specific RNAi fragments (**Table 1**). The polymerase chain reaction (PCR) primers were designed using Premier 5.0 software (**Table 2**). Real-time PCR (RT-PCR) primers for mouse SUMO1 and Ubc9 genes were designed according to their GenBank sequences (**Table 3**). Total RNA was isolated from RAW264.7 cells using an RNA extraction kit (CW Biotech, China) and was reverse transcribed into complementary DNA (cDNA) using the HiFi-Script first-chain synthesis kit (CW Biotech). SUMO1 and Ubc9 genes were PCR-amplified using cDNA as the template. Each target gene was individually ligated into pMD19-T and, after transformation into *Escherichia coli*, positive colonies were picked. The recombinant plasmids containing the SUMO1 or Ubc9 genes were purified. The gene fragments were then isolated by *Hpa*I and *Xho*I digestion and then individually ligated into the pLEX-MCS overexpression vector to generate pLEX-SUMO-1 and pLEX-Ubc9. We also constructed 2 RNAi vectors, pLL3.7-SUMO-1 and pLL3.7-Ubc9, using a similar subcloning strategy.

Generation of stable overexpression and knockdown SUMO1 and Ubc9 cell lines

HEK-293FT cells and RAW264.7 cells were transfected with the lentiviral knockdown or overexpression vectors using Lipofectamine 2000 (Invitrogen, USA) (5 mL of plasmid was added to 2×10^5 cells). The plasmids and cells were mixed thoroughly and placed in a 37°C 5% CO_2 incubator. K-S, O-S, SUMO1 overexpression-knockdown (OS-KS), K-U, O-U, and Ubc9 overexpression-knockdown (OU-KU) groups were generated. After incubation for 48 h, total RNA from each group was extracted using an RNA extraction kit (CW Biotech). The

Table 1. Small interfering RNA fragments

Fragment		Fragment sequences
pLL3.7-SUMO1	F	AACGGAGAATACATTAAACTCAAATTCAAGAGATTTGAGTTTAATGTATTCTCCTTTTTTACGCGTC
	R	TCGAGACGCGTAAAAAAGGAGAATACATTAAACTCAAATCTCTTGAATTTGAGTTTAATGTATTCTCCGTT
pLL3.7-Ubc9	F	AACCCAAAACAGAGTGGAATATGATTCAAGAGATCATATTCCACTCTGTTTTGGTTTTTT ACGCGTC
	R	TCGAGACGCGTAAAAAACCAAAACAGAGTGGAATATGATCTCTTGAATCATATTCCACTCTGTTTTGGGTT

SUMO1, small ubiquitin-related modifier 1; Ubc9, E2 conjugating enzyme 9.

Table 2. Primers sequences

Gene	Primers sequences (5′→3′)	Length (bp)
SUMO-1	F:GGATCCATGTCTGACCAGGAGGCAAA	306
	R:CTCGAGCTAAACCGTCGAGTGACCCCC	
Ubc9	F:GGATCCTCAGCCGCATCTICTITIG	447
	R:CTCGAGGATGGCATGGACTGTGGTC	

Table 3. Primers for real-time quantitative polymerase chain reaction

Gene	Primers sequences (5′→3′)	Length (bp)
β-actin	F:GTGTGATGGTGGGAATGGGT	364
	R:GTCCTTCTGACCCATTCCCA	
SUMO-1	F:ATGTCTGACCAGGAGGCAAAA	306
	R:TTCATTGGAACTCCCTGTCTTT	
Ubc-9	F:GATGAACTGGGAGTGCGCT	477
	R:TTGGATGAAACAGTGGGGG	

cDNA was synthesized from the mRNA by using AMV reverse transcriptase (CW Biotech), following the manufacturer's instructions. The stable cell lines were verified by quantitative RT-PCR (qRT-PCR).

Establishing a cell model for *B. melitensis* 16M infection

To establish a cell model for *B. melitensis* 16M or *Brucella* 16MΔVirB infection, phosphate-buffered saline or lipopolysaccharide-pretreated cells were infected with *B. melitensis* 16M or *Brucella* 16MΔVirB at the logarithmic growth phase with a 100:1 (bacteria: cells) multiplicity of infection. All cell groups (2×10^6 cell/well) were cultured in DMEM containing 10% FBS at 37°C under 5% CO_2. After 1 h, gentamicin (30 g/mL, Sigma-Aldrich, USA) was added to the cell medium for 30 min to kill the extracellular *Brucella*. The cells received different treatments at 0, 4, 8, 12, 24, and 48 h.

Enzyme-linked immunosorbent assay (ELISA) detection of cytokines

At 0, 4, 8, 12, 24, and 48 h after *B. melitensis* 16M infection, cell culture supernatants from the different treatment groups in 12-well plates were collected by *centrifugation* at 3,000 × g for 20 min. Cytokine levels (interferon γ [IFNγ] and tumor necrosis factor α [TNFα]) in the supernatants were measured by performing ELISA (R&D, USA) in accordance with the manufacturer's instructions.

Brucella survival in cells subjected to different treatments

At 0, 4, 8, 12, 24, and 48 h post-infection, cells were collected, lysed, and subjected to gradient dilution, which produced concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6. The bacteria were then evenly coated with *Brucella* solid medium and incubated at 37°C for 72 h followed by colony counting.

qRT-PCR

At 0, 4, and 12 h post-infection, total RNA (10 μg) was isolated from each of the different cell groups and reverse transcribed into cDNA following the manufacturer's protocol. β-actin was used as the housekeeping gene. qRT-PCR was performed using a Light-Cycler 480 (Roche Applied Science, Germany) with the SYBR Premix Ex Taq™ kit (Applied Biosystems, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of the genes (**Table 3**).

Western blotting

At 0, 4, 8, 12, 24, and 48 h post-infection, protein lysate samples were boiled for 10 min in sodium dodecyl sulfate (SDS) buffer. The samples were then separated on a 12% SDS-polyacrylamide gel electrophoresis gel and electro-transferred to a nitrocellulose membrane (Millipore, USA) for 40 min. The membrane was blocked with 5% non-fat milk (Thermo Scientific, USA) for 1 h, and then incubated with rabbit anti-SUMO1 or Ubc9 monoclonal antibodies (Abcam, UK) diluted in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h at 37°C. The membrane was washed with TBST buffer 3 times and incubated with HRP-conjugated mouse anti-rabbit IgG (Bioworld, USA) overnight at 4°C. After 3 washes, the membrane was stained using an HRP-DAB kit (Zhongshan Golden Bridge, China).

Statistical analysis

SPSS Statistics 17.0 software (IBM Corp., USA) was used for data analysis. Independent sample *t*-tests were used for comparisons among the different groups. The experimental data are expressed as mean ± standard deviation values. All tests were repeated three times.

RESULTS

SUMO1 and Ubc9 protein levels decrease following B. melitensis 16M infection

The levels of SUMO1 and Ubc9 proteins in cells infected with *B. melitensis* 16M for 0, 4, 8, 12, 24, and 48 h were determined via western blotting. SUMO1 and Ubc9 levels were decreased from 8 h post-infection onward (**Fig. 1**), indicating that infection with *B. melitensis* 16M inhibited the expression of SUMO1 and Ubc9 from this time onward.

Inhibition of cell viability by knockdown and overexpression plasmids in a time-dependent manner

We tested the effects of knockdown and overexpression plasmids on cell viability by performing MTT assays. The knockdown (pLL3.7-SUMO-1 and pLL3.7-Ubc9) and overexpression (pLEX-SUMO-1 and pLEX-Ubc9) plasmids produced a reduction in cell viability from 48 h after transfection onward (**Fig. 2**).

SUMO1 and Ubc9 gene expression levels in differently treated cells

The relative gene expression levels of SUMO1 and Ubc9 were calculated using the $2^{-\Delta\Delta Ct}$ method (**Fig. 3**). Compared with the control group, SUMO1 expression in K-S cells was significantly decreased ($p < 0.01$), with knockdown efficiency reaching 53%. Ubc9 expression in K-U cells was also significantly decreased ($p < 0.01$), with knockdown efficiency reaching 52%. In the O-S and O-U groups, SUMO1 and Ubc9 expressions were significantly increased after lentiviral infection. The expression levels in the OS-KS and OU-KU groups were also significantly increased ($p < 0.05$). These results indicate that we successfully constructed an experimental platform that included SUMO1 and Ubc9 knockdown, overexpression, and rescue cell lines.
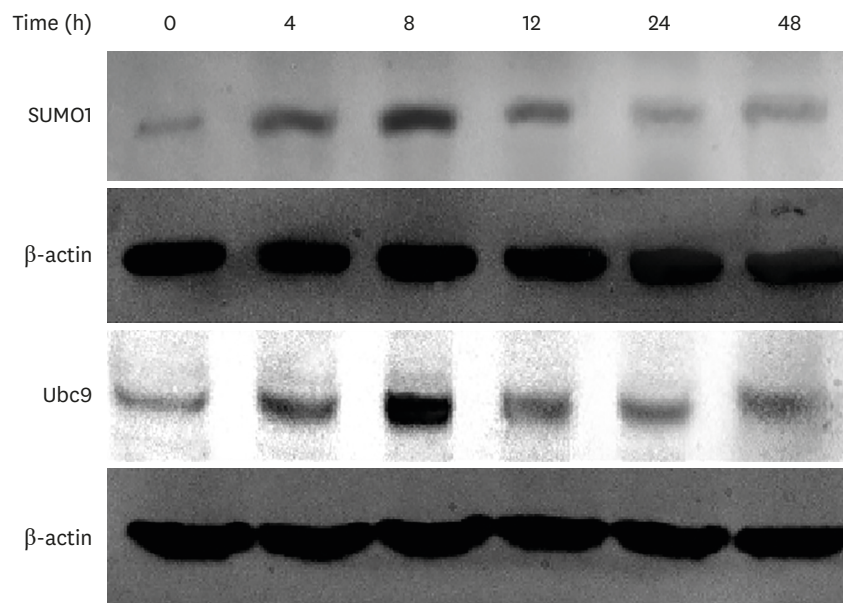

Fig. 1. Western blot results of SUMO1 and Ubc9 in *B. melitensis* 16M-infected normal RAW264.7 cells. Cells were lysed at 0, 4, 8, 12, 24, and 48 h post-infection and proteins extracted. β-actin was the reference gene. SUMO1, small ubiquitin-related modifier 1; Ubc9, E2 conjugating enzyme 9.
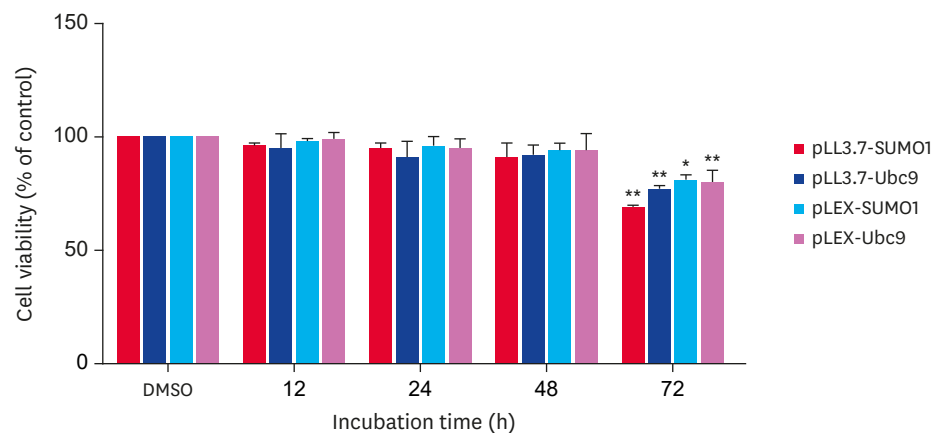
Fig. 2. Cell viability is inhibited by knockdown and overexpression plasmids in a time-dependent manner. Statistical significance is indicated as *$p < 0.05$ or **$p < 0.01$.
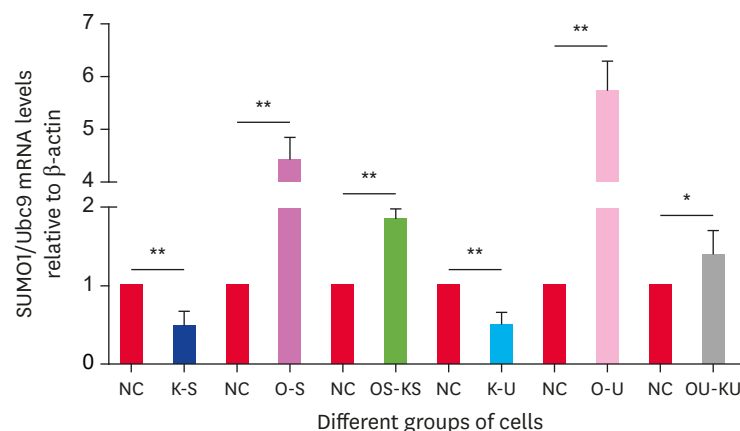SUMO1, small ubiquitin-related modifier 1; Ubc9, E2 conjugating enzyme 9; DMSO, dimethyl sulfoxide.

Fig. 3. Quantitative real-time polymerase chain reaction was performed to analyze the mRNA levels of SUMO1 and Ubc9 in lentivirus-infected RAW264.7 macrophages. Statistical significance is indicated as *$p < 0.05$ or **$p < 0.01$. mRNA, messenger RNA; SUMO1, small ubiquitin-related modifier 1; Ubc9, E2 conjugating enzyme 9; NC, no-treatment cell lines; K-S group, stable SUMO1 knockdown cells; O-S group, cells stably overexpressing SUMO1; OS-KS group, SUMO1 overexpression-knockdown cells; K-U group, stable Ubc9 knockdown cells; O-U group, cells stably overexpressing Ubc9; OU-KU group, Ubc9 overexpression-knockdown cells.

Cytokine production in different RAW264.7 cell treatment groups following infection with *B. melitensis* 16M

Different treatment groups of RAW264.7 cells produced varying levels of IFNγ and TNFα cytokines at different times after infection with *B. melitensis* 16M compared with the control group. At 4, 8, 12, 24, and 48 h after *Brucella* 16M infection, the O-S and O-U groups secreted significantly higher levels of both IFNγ and TNFα compared with the control group ($p < 0.05$), peaking at 12 h post-infection. In contrast, K-S and K-U cells secreted significantly lower levels of IFNγ (**Fig. 4A**) and TNFα (**Fig. 4B**; $p < 0.05$) at 8, 12, 24, and 48 h post-infection compared with the control group ($p < 0.05$). Additionally, the OS-KS and OU-KU groups showed similar significant changes at 12 h post-infection ($p < 0.05$). Collectively, these results indicate that after SUMO1 and Ubc9 silencing, the type 1 T helper (Th1) immune response was decreased in *Brucella*-infected macrophages. Thus, expressions of SUMO1 and Ubc9 appear to benefit the release of Th1 cytokines.
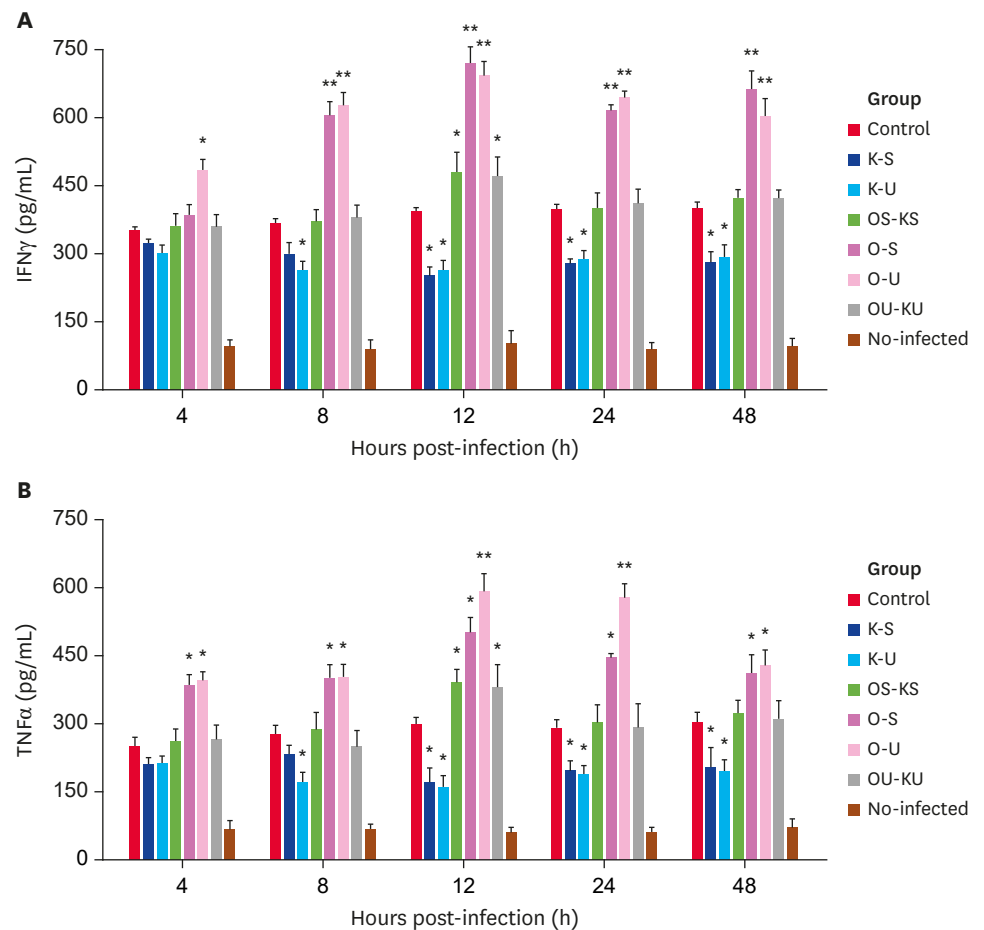
Fig. 4. Enzyme-linked immunosorbent assay detection of IFNγ (A) and TNFα (B) in the supernatants of different treatment groups of RAW264.7 cells after *B. melitensis* 16M infection. *Brucella*-infected-untreated cells acted as the control group. Statistical significance is indicated as *$p < 0.05$ or **$p < 0.01$.

IFN, interferon; TNF, tumor necrosis factor; K-S group, stable SUMO1 knockdown cells; O-S group, cells stably overexpressing SUMO1; OS-KS group, SUMO1 overexpression-knockdown cells; K-U group, stable Ubc9 knockdown cells; O-U group, cells stably overexpressing Ubc9; OU-KU group, Ubc9 overexpression-knockdown cells.

Intracellular survival of *B. melitensis* 16M in RAW264.7 cells after different treatments

We determined whether SUMO1 and Ubc9 affected the intracellular survival of *B. melitensis* 16M in RAW264.7 cells after different treatments (**Fig. 5**). *Brucella* colony forming units (CFU) were counted. From 8 h to 48 h, the O-S and O-U groups had lower *B. melitensis* 16M log CFU values compared with the control group (no treatment) ($p < 0.05$), but the K-S and K-U groups had significantly higher values ($p < 0.05$). The differences between groups were most obvious at 12 h post-infection ($p < 0.01$). The results for the OS-KS and OU-KU groups were essentially the same as those of the control group. These results indicate that inhibition of SUMO1 and Ubc9 proteins promotes *B. melitensis* 16M growth and reproduction.

Effects of the *B. melitensis* 16MΔVirB2 mutant and *B. melitensis* 16M LPS on SUMO1 and Ubc9 expression in mouse macrophages

Western blotting and qRT-PCR results showed that incubation of mouse macrophages with *Brucella* 16M LPS did not induce any change in SUMO1 or Ubc9 expression at any concentration (data not shown). In *B. melitensis* 16MΔVirB2-infected cells, however, western
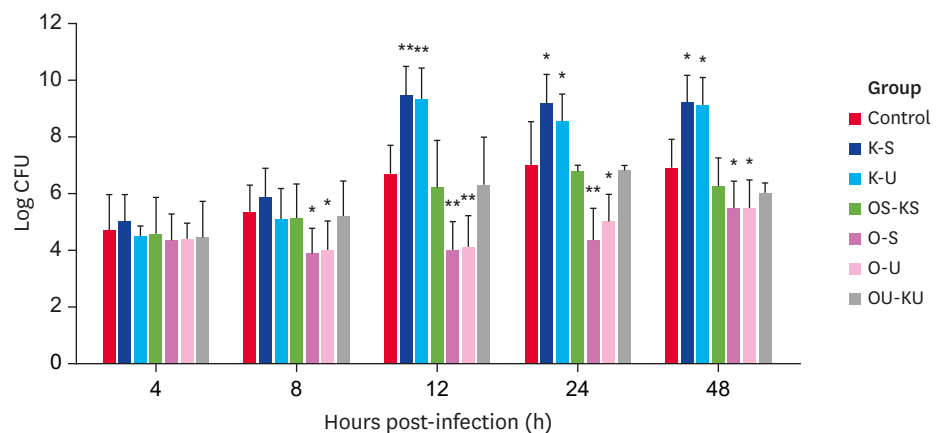
Fig. 5. Intracellular survival of post-infection *B. melitensis* 16M in different RAW264.7 cell treatment groups. RAW264.7 cells were lysed, and *Brucella* was coated with tryptic soy agar culture medium and cultured at 37°C for 72 h. *B. melitensis* 16M numbers were then counted. *Brucella*-infected untreated cells served as the control group. Statistical significance is indicated as *$p < 0.05$ or **$p < 0.01$.
CFU, colony-forming unit; K-S group, stable SUMO1 knockdown cells; O-S group, cells stably overexpressing SUMO1; OS-KS group, SUMO1 overexpression-knockdown cells; K-U group, stable Ubc9 knockdown cells; O-U group, cells stably overexpressing Ubc9; OU-KU group, Ubc9 overexpression-knockdown cells.

blotting and qRT-PCR indicated that the expression levels of SUMO1 (**Fig. 6B**) and Ubc9 (**Fig. 6A**) were significantly increased ($p < 0.05$) at 4 h and 12 h post-infection compared with the control group. This result indicates that the T4SS-VirB2 can negatively regulate SUMO1 and Ubc9 expressions in macrophages, thereby promoting the intracellular survival of *B. melitensis* 16M.

VirB2 regulates intracellular survival of *Brucella* 16M by inhibiting SUMO1 and Ubc9 expression

Western blotting results showed that after incubating cells with VirB protein (from the *Brucella* 16M IV secretion system, T4SS-VirB), Ubc9 protein levels were decreased at 8 h post-infection (**Fig. 7A**). This result was similar to that following *Brucella* 16M infection (**Fig. 1**). In addition, we observed that intracellular survival of the 16MΔVirB2 mutant was significantly decreased at 4 h to 48 h post-infection compared to survival in *Brucella* 16M infection ($p < 0.05$; **Fig. 7B**). A significant increase in colony forming was also observed for VirB2-prestimulated *Brucella* 16MΔVirB2-infected macrophages compared to non-prestimulated *Brucella* 16MΔVirB2-infected macrophages at 12 h and 24 h post-infection. These results indicate that deletion of VirB2 can reduce the virulence of *Brucella* 16M. The VirB2 protein can regulate the expression of Ubc9, thereby increasing intracellular survival of *Brucella* in macrophages at the late stage of infection. Thus, T4SS-VirB2 is a key virulence protein that benefits intracellular survival and replication of *Brucella* 16M.

DISCUSSION

This study aimed to investigate whether SUMO1 and the enzyme responsible for its conjugation to target proteins, Ubc9, regulate the host immune reaction, apoptosis, and subsequent protection against intracellular survival of *Brucella* 16M in macrophages. *Brucella* can maintain a long-lasting interaction with host cells, depending on the secretion of various virulence factors that modulate host cell apoptosis, immune factor production, and antigen presentation [11,12]. The *Listeria monocytogenes* listeriolysin O virulence factor can intentionally or unintentionally interfere with SUMOylation by degrading Ubc9 [13].
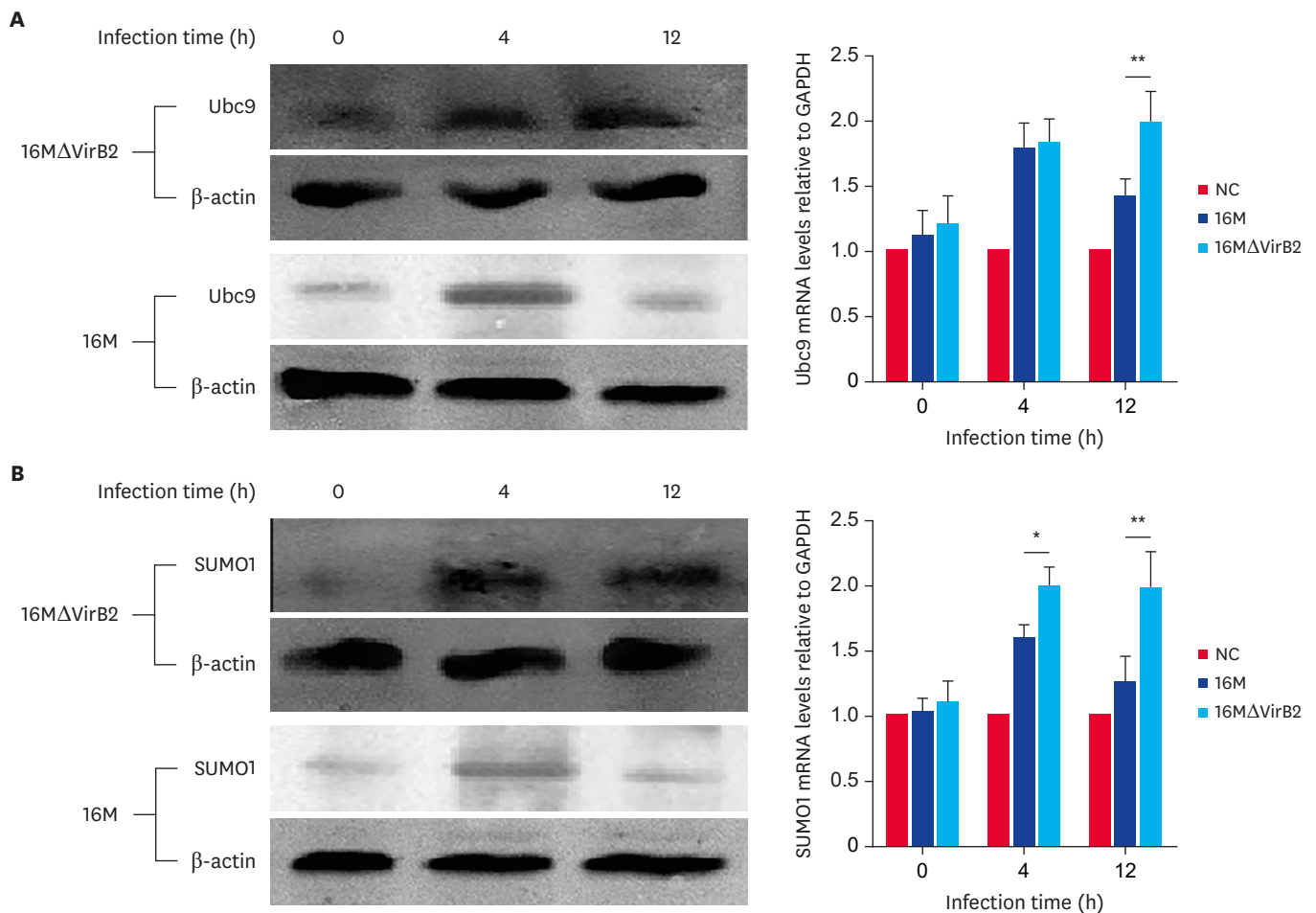
Fig. 6. Effect of T4SS-VirB2 on expression of SUMO1 and Ubc9 after infection of RAW264.7 cells with *B. melitensis* 16M or *B. melitensis* 16MΔVirB mutant. Western blotting was used to detect the protein levels of SUMO1 and Ubc9 (A). Quantitative real-time polymerase chain reaction was conducted to detect the levels of SUMO1 and Ubc9 mRNAs (B). Statistical significance is indicated as $^*p < 0.05$ or $^{**}p < 0.01$.
NC, no-treatment cell lines; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; SUMO1, small ubiquitin-related modifier 1; Ubc9, E2 conjugating enzyme 9.
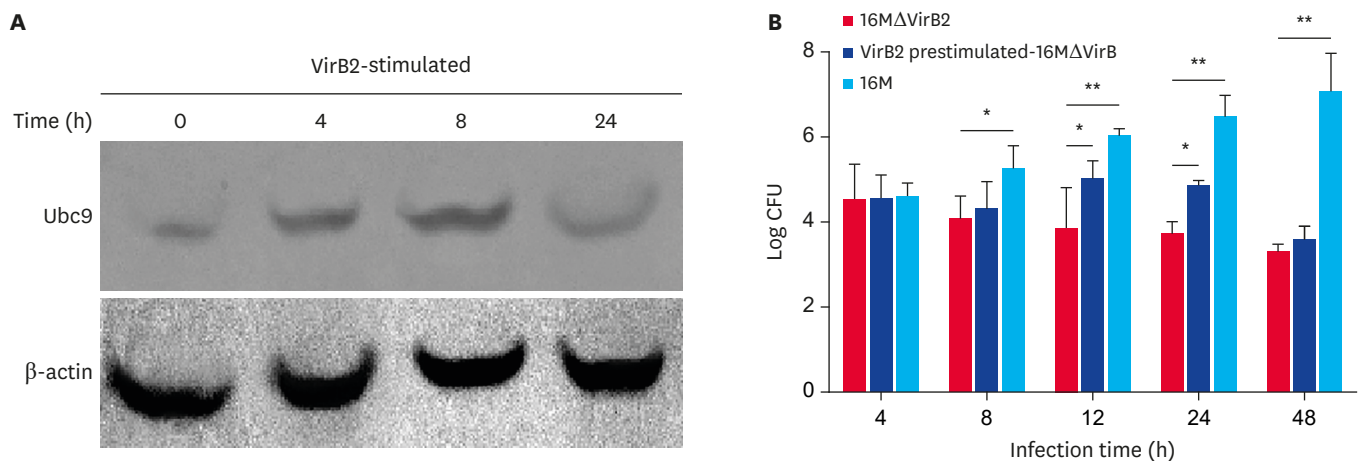

Fig. 7. VirB2 affects intracellular survival of *Brucella* 16M by inhibiting Ubc9 expression. Levels of Ubc9 after pre-stimulation with VirB2 for 0, 4, 8, and 24 h were assessed by western blotting (A). *Brucella* 16M and *Brucella* 16MΔVirB2 cells pre-stimulated or non-prestimulated with VirB2. At 4, 8, 12, 24, and 48 h post infection, the number of bacteria in cells was counted (B). Statistical significance is indicated as $^*p < 0.05$ or $^{**}p < 0.01$.
CFU, colony-forming unit; Ubc9, E2 conjugating enzyme 9.

SUMOylation can regulate the IFNγ-activated immune response [14]. Considering that *Brucella* and *L. monocytogenes* are both intracellular bacteria, their pathogenic mechanisms may be similar; therefore, investigation of the interaction between *B. melitensis* 16M and SUMO1/Ubc9 in macrophages may help to unravel the invasion and intracellular survival of *Brucella* 16M. In this study, we confirmed that *Brucella* 16M can affect the host immune response and host cell apoptosis by interfering with the SUMOylation system (SUMO1/Ubc9). This benefits *Brucella* intracellular survival at the late stage of infection. VirB2, a key *Brucella* 16M virulence factor, can decrease SUMO1/Ubc9 expression, thereby helping *Brucella* 16M avoid death by the host immune system.

SUMOylation has a critical role in many cellular processes, such as immune response and cell proliferation [15]. Cytokines mediate and direct the immune response and are important factors in protection against brucellosis [16,17]. In this study, we found that *Brucella* 16M can strongly activate SUMO1 and Ubc9 expression and can upregulate immune factors at the early stage of infection. Knockdown and overexpression of these two genes directly affected the classical Th1 immune response (IFNγ and TNFα) in *Brucella* 16M-infected RAW264.7 macrophages (**Fig. 4**). IFNγ is a key Th1-type immune cytokine that is required for the bactericidal activity of macrophages [18]. TNFα is one of the most important pro-inflammatory and pro-immune cytokines involved in type-1 cell-mediated immune responses to intracellular pathogens, and it positively controls the early production of IL12 and IFNγ in *Brucella*-infected mice [19]. The absence of TNFα/IFNγ production must favor the early establishment of the bacteria in host cells. Previous studies have suggested that over 90% of *Brucella* internalized by macrophages are cleared by immune cells at the early stage of infection; however, a few *Brucella* can survive by evading host immunity and can become established and grow in the endoplasmic reticulum [20]. We suggest that because the host can produce a strong immune response to kill *Brucella* in the early stage of infection by activating SUMO1/Ubc9, *Brucella* avoids host immune killing in the late stage of infection by inhibiting SUMO/Ubc9 expression. This reduces the release of SUMO1/Ubc9-mediated immune cytokines and weakens the host's ability to remove *Brucella*. In this way, *Brucella* can maintain β-1,2-glucans, the type IV secretion apparatus, and sensing and regulatory systems that allow the bacteria to reach a safe intracellular niche in the endoplasmic reticulum before an effective immune reaction is developed [21]. Given that the humoral immune response, in part mediated bySUMO1/Ubc9, has a pivotal role in controlling *Brucella* infection, we evaluated the number of intracellular *Brucella* 16M. The result confirmed that the intracellular survival of *Brucella* 16M is indeed closely related to the expression of SUMO1/Ubc9. Thus, *Brucella* improves its intracellular viability by inhibiting the SUMO1/Ubc9-mediated release of immune factors, which also promotes persistence in *Brucella* infection *in vivo*.

To further analyze the role of SUMO1/Ubc9 in the survival and proliferation of *Brucella* 16M in monocytes, we investigated whether *Brucella* 16M infection affects (induces or prevents) spontaneous or stimulated apoptosis of host cells via the SUMO-mediated release of immune factors. We found that *Brucella* 16M can reduce the level of IFNγ by inhibiting the expression of SUMO1/Ubc9. *Brucella* infection renders macrophage-like cells resistant to Fas ligand- or IFNγ-induced apoptosis [22]. This indicates that *Brucella* not only inhibits the expression of immune factors but also promotes the intracellular survival of bacteria by inhibiting IFNγ-induced apoptosis. Furthermore, previous experiments have confirmed that the release of IFNγ is regulated by SUMO1/Ubc9. By knocking down or overexpressing SUMO1/Ubc9 genes, SUMO1/Ubc9 gradually affected (promoted/inhibited) apoptosis at 24 h after *Brucella* 16M infection, as shown in **Supplementary Fig. 1**. Therefore, we suggest that *Brucella* 16M can

reduce apoptosis by reducing SUMO expression at the late stage of infection. Other studies have also reported that SUMO is involved in the regulation of apoptosis [23]. The ability of *Brucella* to escape host immune killing and survive in cells mainly depends on secreting various effector proteins or virulence factors that inhibit apoptosis and the release of immune factors [11]. Thus, inhibition of apoptosis might represent a beneficial strategy for *Brucella* development in infected hosts. The result presented in **Fig. 4** indicates that the expression of SUMO1/Ubc9 determines the intracellular survival of Brucella 16M, especially in the late stage of infection. Together, our analyses indicate that this is because *Brucella* infection inhibits SUMO-mediated IFNγ release, thereby inhibiting IFNγ-mediated apoptosis, which promotes the intracellular survival of *Brucella*. This may be another important cause of chronic *Brucella* infection *in vivo*. Previous research attention has focused on revealing the mechanism by which host apoptotic genes are regulated under *Brucella* infection at proteome and transcriptome levels [24]. However, the mechanism by which *Brucella* inhibits macrophage apoptosis has not been determined. Therefore, we speculate that our conclusion may help elucidate the relationship between *Brucella* infection and apoptosis.

However, what components of *Brucella* 16M affect the expression of SUMO1 and Ubc9? As we know, *Brucella* 16M is a virulent strain that expresses a complete T4SS and LPS. *Brucella* LPS, along with 32 other virulence factors, are key to *Brucella* infection [25]. In this study, *Brucella* 16M LPS did not induce any changes in SUMO1 or Ubc9 expression in mouse macrophages at any concentration. Thus, LPS did not interact with SUMO1/Ubc9 during *Brucella* infection. The T4SS complex is comprised of a family of proteins, and with its help, bacteria can secrete effector molecules in host cells when they cross the cell membrane [26]. *Brucella*, unlike other gram-negative pathogens, does not have a typical type III secretory system. Instead, it secretes virulence factors in host cells mainly through the T4SS complex. The *Brucella* T4SS complex is comprised of 12 proteins (VirB1–12) that are encoded by VirB operons [26]. VirB2 is a *Brucella* surface protein. When VirB2 is deleted, the ability of *Brucella* to survive in mice is significantly reduced [27]. We found that virulence and intracellular viability of *B. melitensis* 16MΔVirB2 were significantly decreased compared those of *B. melitensis* 16M. Interestingly, expression levels of SUMO1 and Ubc9 were significantly increased in *B. melitensis* 16MΔVirB2 12 h after infection ($p < 0.05$). We, therefore, speculate that T4SS/VirB2 directly interacts with SUMO and Ubc9, which interferes with SUMOylation. We then incubated macrophages with purified VirB2 protein and observed that VirB2 can activate Ubc9 similarly to that from *Brucella* 16M in the early stage of infection. A strong immunity can inhibit the development of a persistent *Brucella* infection [28]. However, in the late stage of infection, VirB2 can inhibit Ubc9 expression. Therefore, we analyzed whether stimulation of VirB2 can rapidly and spontaneously induce a SUMO1/Ubc9-mediated natural immune response to remove the bacteria. We incubated cells with VirB2 and then infected them with the *B. melitensis* 16MΔVirB2 mutant. This improved the intracellular survival of *Brucella* compared with no VirB2 stimulation. Overall, VirB2 of *Brucella* T4SS is a key virulence protein that promotes survival and reproduction in host cells by inhibiting SUMO/Ubc9 modification at the late stage of infection.

In conclusions, we report that inhibition of SUMO1/Ubc9 expression by *Brucella* 16M is a strategy for its intracellular survival. Overexpression of SUMO1 or Ubc9 increases the host's immune response and apoptosis, and it inhibits *Brucella* 16M survival in macrophages. Silencing of SUMO1 or Ubc9 genes inhibits the host's immune response and apoptosis and promotes *Brucella* 16M survival in macrophages. We conclude that *Brucella* 16M can inhibit the release of SUMO-mediated immune factors and apoptosis by reducing the expression

of SUMO1 and Ubc9 genes. This reduction promotes escape from immune killing and intracellular survival. In addition, VirB2 may be a key virulence factor of *Brucella* that inhibits the expression of SUMO1/Ubc9 at the later stage of infection. This study provides a theoretical basis for exploring the molecular mechanism of persistent *Brucella* infection.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Evaluation of apoptosis after *B. melitensis* 16M infection in different groups of RAW264.7 cells. The RAW264.7 cell overexpression and knockdown model was used for infection with *B. melitensis* 16M. Cells were collected at 4, 12, and 24 h post-infection, and V-FITC/PI annexin double-staining with flow cytometry was used. The results represent the mean values from 3 experiments ± standard deviation. Statistical significance is indicated as $^*p < 0.05$ or $^{**}p < 0.01$.

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