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RESEARCH ARTICLE

Ubiquitin-like protein SUMO-1 is essential for the survival of *Brucella melitensis* 16M inside RAW264.7 macrophages

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ABSTRACT

Brucellosis is a globally distributed zoonotic disease that causes animal and human diseases. *Brucella* pathogenesis depends on their ability of inhibiting apoptosis and establishes a replicative niche inside host cells. SUMOylation is a major regulator of protein function that is essential for some pathogenic bacteria during infection. However, the relationship between *Brucella* and SUMOylation remains largely unknown. In our report, we demonstrated that the *Brucella melitensis* 16M (16M) infection leads to a decrease in the expression of SUMO1 proteins with time-delay, and 16M intracellular replication was inhibited by SUMO-1 overexpression and promoted by SUMO-1 depletion, and 16M-dependent apoptosis and the secretion of gamma interferon and interleukin-6 were induced by SUMO-1 overexpression and restricted by SUMO-1 depletion in the RAW264.7 macrophages. Together, this study shows that SUMO-1 plays an essential role in regulating 16M infection and may help to unravel pathogenic mechanisms of 16M.

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INTRODUCTION

Brucella spp are facultative intracellular bacteria that can multiply within the specialized phagosome of both humans and animals, resulting in heavy economic losses and human suffering (Lacerda *et al*., 2010; Iqbal *et al*., 2013). *Brucella spp* cause acute, transmissible infections in animal reservoirs and abortion in pregnant animals (Ficht, 2003; Gul *et al*., 2013). The human disease is also caused by *Brucella melitensis*, *Brucella abortus* or *Brucella suis*. Infected humans can have fever, arthritis, spondylitis, dementia, and meningitis or endocarditis on rare circumstances (Hamdy *et al*., 2002; Elzer *et al*., 2002; Godfroid *et al*., 2005). Currently, there is no effective or safe *Brucella* vaccine for humans and animals, and the physiopathology of brucellosis remains poorly understood.

Small ubiquitin-related modifier (SUMO) was first identified in the mid-1990s as a protein that covalently linked to a target protein post-translationally (Matunis *et al*., 1996; Sun *et al*., 2011). There are major three SUMO isoforms, SUMO 1-3 (Feng *et al*., 2013). SUMO2 and SUMO3 are 95% similar to each other, but they have only 50% identity with SUMO1 (Geiss-Friedlander and Melchior, 2007). SUMO 1-3 is conjugated to different target proteins and has different functions in specific organs or tissues. SUMOylation plays an important role in the regulation of many cell processes such as transcription and replication (Geiss-Friedlander and Melchior, 2007). The SUMOylation system may contribute to an intrinsic defense mechanism against microbial challenge, but the relationship between *Brucella* and SUMOylation remains largely unknown.

In this paper, we investigated whether *Brucella* was able to modify SUMO1 of RAW264.7 macrophages, and the role of SUMO1 protein overexpression and SUMO-1 depletion on the growth of RAW264.7 macrophages. These results indicated for the first time that there was a decrease in expression of SUMO1 protein caused by *Brucella* infection, and 16M intracellular replication was inhibited by SUMO-1 overexpression and promoted by SUMO-1 depletion and *B. melitensis*-dependent apoptosis was induced by SUMO-1 overexpression and restricted by SUMO-1 depletion in the RAW264.7 macrophages.

MATERIALS AND METHODS

Cell culture: Mouse macrophage RAW264.7 cells were purchased from Type Culture Collection of the Chinese

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Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were cultured at 37°C in a humidified 5% CO2.

***Brucella* infection:** Mock vector-transfected cells, pSUMO1-transfected cells, or parental cells were infected with the 16M. Briefly, 2×10^6 cells/well was grown in a 6-well plate for 36 h at 37°C. RAW264.7 cells infected with *Brucella* at 100 MOI. At 1 h post-infection, the cells were washed thrice with PBS and then incubated with gentamicin (50 μg/ml) for 40 min to kill extracellular bacteria. Then, the culture was replaced with DMEM containing gentamicin (25 μg/ml). At 0, 4, 8, 12, and 24 h post-infection, the cells were lysed and viable bacteria were enumerated on TSA plates. The levels of IFN-γ and IL-6 in the supernatants were measured with an ELISA kit (R&D Systems). The data were obtained from three independent experiments.

Expression vector construction and cell transfection: The obtained SUMO1 cDNA was amplified by using primers as follows: primer sense, 5' AGATCTCATGTCT-GACCAGGAGGCAA 3', and primer antisense, 5' GTCGACCTAAACCGTCGAGTGACCC 3'. The enzyme sites for Bgl II and Sal Iam underlined. The pcDNA3.1-SUMO1 plasmid was generated by insertion of a 306-bp fragment of the full-length *Mus musculus* SUMO1 cDNA into the Bgl II/Sal I sites of the pcDNA3.1 vectors. The pcDNA3.1 mock vectors (as a blank control) and pcDNA3.1-SUMO1 plasmid were transfected using Lipofectamine 2000 (Invitrogen) into RAW264.7 cells. After 48 h, the medium was replaced with DMEM containing G418(500 μg/ml; Geneticin, Sigma).After fourteen days, positive colonies were obtained and cells were re-suspended in medium followed by culture, aiming to achieve cells with stable expression of SUMO1.

RNAi experiments: To construct SUMO1 shRNA against mouse SUMO1 mRNA, oligonucleotides 5'-gatccGACA-GGGAGTTCCAATGAAAATTCAAGAGATTTTCATTG GAACTCCCTGTCTTTTTTACGCGTg-----3'(top strand) and 5'-aattcACGCGTAAAAAAGACAGGGAGTTCCA-ATGAAAATCTCTTGAATTTTCATTGGAACTCCCTG TCg-----3' (bottom strand) were annealed together to produce a double-stranded DNA, which was then cloned into RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (Clontech, BD Biosciences) at the BamHI and EcoRI sites. Fluorescent, nonsilencing 21-bp double-stranded RNA (Qiagen) was used as a control RNA (control RNAi). Constructs of the recombinant and control RNAi were then transfected into RAW264.7 cells using Lipofectamine 2000 (Invitrogen). RAW264.7 cells grown to about 70-80% confluence were incubated in six-well plates with 2 ml/well serum-free DMEM containing Lipofectamine 2000 (8 μl) and pSIREN-siRNA (5 μg). At 8 h post-transfection, the cells were replenished with DMEM and incubated for an additional 48 h.

MTT assay: RAW264.7 cells were seeded in 96-well plates (5×10^4 cells/ml) and cells were washed thrice with PBS at 0, 12, 24 and 48 hours after transient transfection of pcDNA3.1-SUMO1 or pcDNA3.1 mock vectors, and were assessed by cell viability using MTT assay (Sigma). MTT (50 μl/well) was added and incubated at 37°C for 4 h. Then the supernatants were removed and 100 μl of dimethyl sulfoxide (DMSO) (Sigma, USA) was added to each well. Optical density was measured by eluting the dye with DMSO, and OD570 was determined. The data were obtained from three independent experiments.

Assessment of apoptosis: Apoptotic rates were evaluated by an Annexin V-EGFP Apoptosis Detection Kit (Bestbio, Shanghai). Annexin V/PI double-positive staining and fluorescence intensity measurements were performed on RAW264.7 cells. The extent of apoptosis was determined using FASC Aria II flow cytometer (BD Biosciences).

Western blot analysis: To determine the SUMO1 expression in RAW264.7 macrophages, cells were lysed in ice-cold RIPA lysis buffer for 20 min and centrifuged at 12,000 rpm for 20 min at 4°C. The protein samples were boiled for 5 min and 10 μl samples was subjected to 12% SDS-PAGE. The protein was electro-transferred onto nitrocellulose membrane (Bio-Rad) at 200 mA for 30 min. Unbound sites on the membrane were blocked with 2% BSA in borate-buffered saline for 1h at 37°C. After washing the membrane three times with TBST buffer for 30 min, then incubated overnight at 4°C with a 1/1500 dilution of rabbit anti-mouse anti-SUMO-1 pAb polyclonal antibody (Bioworld, Minneapolis, USA). The membrane was incubated with goat anti-rabbit IgG (peroxidase conjugated) for 1 h at 37°C after being washed three times. Bound conjugate was visualised with ECL (Thermo Fisher Scientific, USA) after further washing.

Quantitative Real-Time PCR analysis: RAW264.7 cells were infected with 16M according the above method. Total RNA (10 μg) was extracted and amplified with AMV reverse transcriptase (TaKaRa, Japan) following the manufacturer's instructions. The Real-time PCR was conducted on Light-Cycler 480 (Roche Applied Science) with SYBR Premix Ex TaqTM reagent Kit (TaKaRa, Japan). PCR primers for p53 were sense 5' GTACCTTATGAGCCACCCGA 3' and anti-sense 5' GCACAAACACG-AACCTCAAAG 3'. The primer pairs for GAPDH gene were sense 5' CTGCCCAGAACATCA-TCCCT 3' and anti-sense 5' GACACATTGGGGGTAGG-AAC 3'. The relative level of p53 mRNA was calculated by 2-ΔΔCt Method.

Statistical analysis: Student's t-test was used for comparisons among different groups. P values of <0.05 were considered statistically significant.

RESULTS

Decrease in SUMO1 protein: The expression of SUMO1 protein was determined by Western blot in *B. melitensis*-infected macrophages at 0h, 4h, 12h, and 24h. The 16M-infected cells displayed a trend of decreased expression of SUMO1 proteins at the observation time (Fig. 1). The results showed that 16M inhibited the expression of SUMO1 protein in a time-dependent manner.

Effect on 16M replication: Western blot analysis was performed to analyze the overexpression or depletion of

SUMO1 protein. As shown in Figure 2A, the higher level of SUMO1 protein was detected in pSUMO1 cells when compared with that in parental cells (RAW264.7 macrophages) or control cells which were transfected with empty vector pcDNA3.1 and the level of SUMO1 protein was markedly reduced in cells transfected with SUMO1 siRNA compared with cells transfected with control siRNA (Fig. 2B).

We next evaluated whether the overexpression and depletion of SUMO1 protein has any influence on 16M intracellular growth. At 4 h post-infection, there was no difference (P>0.05) at the levels of 16M replication in all groups of cells (Fig 2C). At 12 h post-infection, a 1.01-log decrease (P<0.05) in the number of 16M inside pSUMO1-transfected cells while a 0.68-log increase (P<0.05) in that for SUMO1 RNAi-transfected cells compared to that for parental cells (Fig. 2C). At 24 h post-infection, the difference was even greater at 1.65-log and 1.12-log, respectively (P<0.01; Fig. 2C). We observed similar levels of intracellular infection among mock vector-transfected cells, parental cells, and control RNAi-transfected cells. These results showed that 16M infection was restricted by SUMO1 overexpression and promoted by SUMO1 RNAi in the RAW264.7 macrophages.

Effect on macrophages growth: We next investigated whether overexpression and depletion of SUMO1 protein affect the growth of RAW264.7 macrophages. The viability of RAW264.7 cells transfected with pSUMO1 and SUMO1 RNAi was analyzed by MTT assay at 0h, 12h, 24h, and 48h. There was not a loss of viability of the pSUMO1-transfected macrophages compared with that of parental or mock vector-transfected macrophages (data not shown). Relative to the control, SUMO1 RNAi did not lead to increased cell death (data not shown). These results suggest that overexpression and depletion of SUMO1 do not affect the growth of RAW264.7 cells.

Role in apoptosis: The 16M-dependent apoptosis rates of pSUMO1-transfected cells were analyzed by flow cytometry at 0h, 12h and 24h compared with mock vector-transfected cells or parental cells. Induced by 16M infection, the apoptotic rate of pSUMO1-transfected cells had a rang of 18.38 to 36.83% and that of mock vector-transfected cells or parental cells had a rang of 14.97 to 23.86% or 15.34 to 23.16% (Fig. 3A), and the apoptotic rate of SUMO1 RNAi-transfected cells had a rang of 15.17 to 18.11% (Fig. 3A). The 16M-dependent apoptotic rates of SUMO1 overexpression cells were significantly higher than rates in both mock vector-transfected cells and parental cells at all times (P<0.01) and the differences have a trend of increase with time delay, and that of SUMO1 RNAi-transfected cells were significantly lower than rates in both control RNAi cells and parental cells at 12 or 24 h post-infection, while similar levels (P>0.05) of apoptotic rates existed in mock vector-transfected cells, parental cells, and control RNAi cells. The tumor suppressor protein p53 involves in the cellular responses to a variety of stress signals and plays a key role in promoting apoptosis process, which is linked with SUMO1 protein. Therefore, real-time PCR was also used to measure p53 mRNA expression in SUMO1- over-expressing, SUMO1 RNAi cells, and control cells. Compared to control cells, levels of p53 gene were

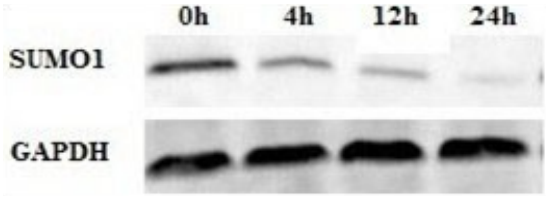

Fig. 1: Decrease in SUMO1 protein upon infection with *B. melitensis* 16 M. Western blot analysis of SUMO1 protein from RAW264.7 macrophages infected for 0h, 4h, 12h, and 24h with 16M. GAPDH levels are shown as a loading control.

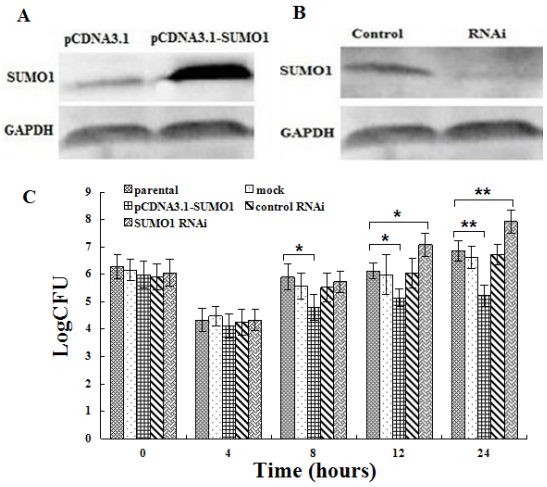

Fig. 2: (A) Western blot analysis of RAW264.7 macrophages overexpressing SUMO1 protein compared with respective control cells; a blank plasmid (pcDNA3.1[+]) was used as control. (B) Western blot analysis of SUMO1 expression can be suppressed by RNA silencing compared with cells transfected with control siRNA. Detection of GAPDH served as a loading control.(C) Effect of SUMO1 overexpression and SUMO1 depletion on intracellular replication of 16M within RAW264.7 macrophages. Significant differences of 16M number inside pSUMO1-transfected cells and SUMO1 RNAi-transfected cells compared to that of parental cells indicated as follows: *P<0.05; **P<0.01.

significantly higher (P<0.01) in the 16M-infected SUMO1 overexpressing cells and was significantly lower (P<0.01) in the 16M-infected SUMO1 RNAi cells 12 or 24 h post-infection (Fig. 3B). The Real-time PCR results showed that SUMO1 overexpression and SUMO1 RNAi affected the expression of apoptosis-related gene p53 in the 16M-infected macrophages, which provided independent confirmation of the flow cytometry analysis. These results showed that 16M-dependent apoptosis was induced by SUMO1 overexpression and inhibited by SUMO1 RNAi in the 16M-infected RAW264.7 macrophages.

Cytokine response: The levels of IFN-γ (Fig. 4A) and IL-6 (Fig. 4B) were assessed in supernatants of 16M infected cells under the conditions of SUMO1 overexpression or depletion. SUMO1 overexpressing cells produced significant higher levels of IFN-γ and IL-6 at 12 and 24h post-infection compared to mock vector-transfected or parental cells (P<0.01), and SUMO1 RNAi cells exhibited significant lower levels of IFN-γ and IL-6 compared to control RNAi or parental cells (P<0.05). These results indicated that classical Th1 and Th2 responses are elicited by SUMO1 overexpression and inhibited by SUMO1 RNAi in the 16M-infected RAW264.7 macrophages.

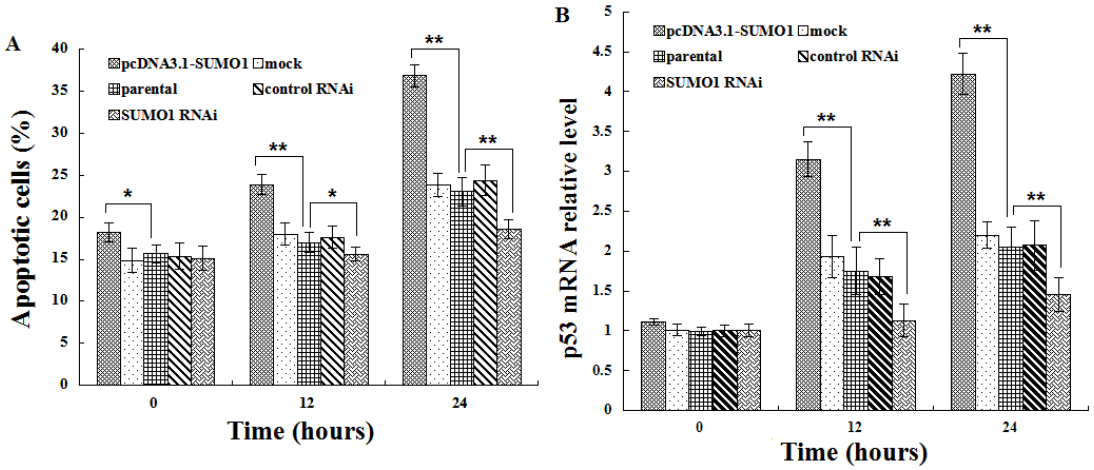

Fig. 3: (A) 16M-dependent apoptosis analysis of transfected RAW264.7 macrophages with Annexin V/PI double staining detected by a flow cytometer. (B) Relative level of p53 mRNA was measured by real-time PCR. Significant differences of 16M-dependent apoptotic rates and p53 mRNA in pSUMO1-transfected cells and SUMO1 RNAi-transfected cells compared to that of parental cells indicated as follows: *P<0.05; **P<0.01.

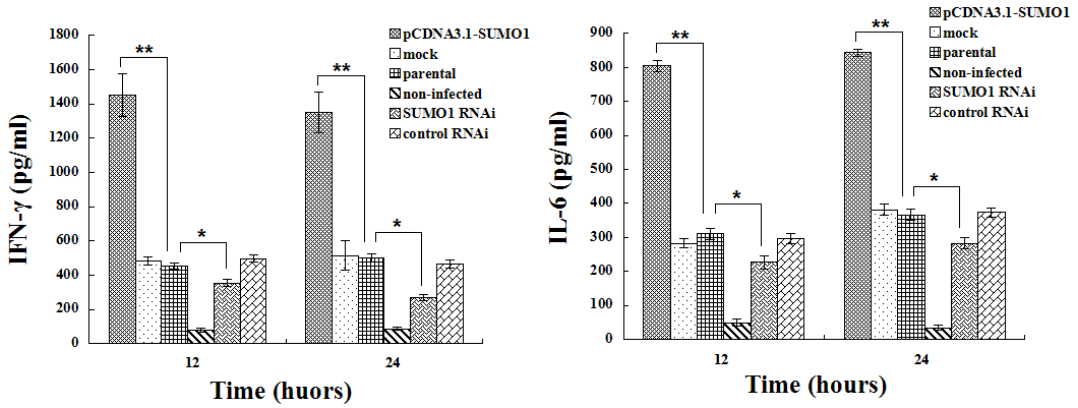

Fig. 4: The analysis of IFN-γ (A) and IL-6 (B) secretion was measured by ELISA from the supernatant of 16M infected SUMO1-overexpressing or SUMO1 RNAi-transfected cells. Significant differences of pSUMO1-transfected cells and SUMO1 RNAi-transfected cells compared with parental cells indicated as follows: *P<0.05; **P<0.01.

DISCUSSION

More than 90% of *Brucella* internalised by macrophages are killed after phagocytosis, but a few *Brucella* can evade host immunity and establish an intracellular niche permissive for growth (Bargen *et al*., 2012). Pathogenic bacteria interfere with the SUMOylation system of host cell for achieving infection, and some pathogens have developed several strategies to act directly on the ubiquitination pathway through mimicking host cell proteins (Chosed *et al*., 2007) or indirectly by interfering with the ubiquitination pathway (Spallek *et al*., 2009). Closely related post-translational modifications involve covalent links to Ub-like proteins (Stulemeijer and Joosten, 2008), such as the SUMO-1 to-4 (Dupont *et al*., 2010), which were activated during pathogen infection. It has been reported that the protein SUMOylation was reduced globally by *Listeria* infection, and LLO (listeriolysin O) activity of *Listeria* plays an important role in the process (Ribet *et al*., 2010). In addition to LLO, other bacterial pore-forming toxins including Perfringolysin O of *Clostridium perfringens* and Pneumolysin of *Streptococcus pneumoniae* can trigger the degradation of Ubc9 (Ribet *et al*., 2010). Moreover, viruses can also interfere with the host cell SUMOylation (Spallek *et al*., 2009). The *Brucella* T4SS is encoded by virB operon 12 open reading frames (virB1 to virB12) (O'Callaghan *et al*., 1999), which is essential to the virulence of all *Brucella* strains and plays an important role in establishment of the *Brucella* replication niche (Boschiroli *et al*., 2002). Our study showed that *Brucella melitensis* 16M could lead to a decrease in the level of SUMO1 protein. After internalization, we speculate that *Brucella* T4SS may involve in decrease with the level of SUMO1 protein. Therefore, further testing will be needed to determine whether T4SS plays a role in impairing the SUMO1 protein by *Brucella melitensis* infection.

The Th1 immune responses characterized by IFN-γ production are related to the host immune response to *Brucella*, which are activated by vaccines (Golding *et al*., 2001). Previous studies showed that IFN-γ is a critical cytokine required for macrophage bactericidal activity (Sathiyaseelan *et al*., 2006). IL-6 is a Th2 cytokine considered as an anti-inflammatory mediator. Thus, the mixed Th1/Th2 responses can be obtained through the detection of IFN-γ and IL-6. The number of intracellular viable bacteria was evaluated to detect the role of overexpression and depletion of SUMO1 during *Brucella*

melitensis infection. The results showed that *Brucella melitensis* 16M survival inhibited by SUMO1 over-expression and promoted by SUMO1 depletion, and we speculate that this may be associated with mixed Th1/Th2 responses.

Brucella can survive and replicate for long periods within host cells which depend on producing various virulence factors that change apoptosis, cytokine secretion, phagocytosis, phagolysosome fusion, and antigen presentation (Vrioni *et al*., 2008). However, the macrophage can induce its own apoptosis to avoid the multiplication of intracellular bacteria (Skendros *et al*., 2011), virulent *Brucella* strains inhibit macrophage apoptosis for favoring their survival and replication (Gross *et al*., 2000; Fernandez-Prada *et al*., 2003). SUMO may regulate apoptosis by modulating Hipk subcellular localization or the activity of the tumor suppressor protein p53 (Smith *et al*., 2012). SUMO needs to be present at the site of Dmp53 action, which does not change p53 activity through a direct effect (Mauri *et al*., 2008; Pardi *et al*., 2011). Like mammalian p53, Dmp53 appears to be a target of SUMOylation and plays an important role in induction of apoptosis. The results showed that SUMO1 protein overexpression could induce *B. melitensis*-dependent apoptosis, which confer host cells resistance to survival and replication of *B. melitensis*.

Conclusion: This is the first study about the role of SUMO1 protein on *B. melitensis* 16M survival in the RAW264.7 macrophages. Our results suggest that the survival of *B. melitensis* 16M was inhibited by SUMO1 overexpression and promoted by SUMO1 depletion, and *B. melitensis*-dependent apoptosis was induced by SUMO1 overexpression and inhibited by SUMO1 depletion, which is important for discovering the novel treatment for chronic brucellosis in livestock and humans. The molecular mechanisms underlying the interaction between the *B. melitensis* 16M and the SUMOylation of host remain to be clarified.

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REFERENCES

Bargen K, JP Gorvel and SP Salcedo, 2012. Internal affairs: investigating the *Brucella* intracellular lifestyle. FEMS Microbiol Rev, 36: 533-562.

Boschiroli ML, S Ouahrani-Bettache, V Foulongne, S Michaux-Charachon, G Bourg, A Allardet-Servent, C Cazevieille, JP Liautard, M Ramuz and D O'Callaghan, 2002. The *Brucella suis* virB operon is induced intracellularly in macrophages. Proc Natl Acad Sci USA, 99: 1544-1549.

Chosed R, DR Tomchick, CA Brautigam, S Mukherjee, VS Negi, M Machius and K Orth, 2007. Structural analysis of Xanthomonas XopD provides insights into substrate specificity of ubiquitin-like protein proteases. J Biol Chem, 282: 6773-6782.

Dupont N, N Temime-Smaali and F Lafont, 2010. How ubiquitination and autophagy participate in the regulation of the cell response to bacterial infection.Biol Cell, 102: 621-634.

Elzer PH, SD Hagius, DS Davis, VG DelVecchio and FM Enright, 2002. Characterization of the caprine model for ruminant *brucellosis*. Vet Microbiol, 90: 425-431.

Feng ZJ, B Gurung, GH Jin, XL Yang and XX Hua, 2013. SUMO modification of menin. Am J Cancer Res, 3: 96-106.

Fernandez-Prada CM, EB Zelazowska, M Nikolich, TL Hadfield, RM Roop, GL Robertson and DL Hoover, 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O-Polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect Immun, 71: 2110-2119.

Ficht TA, 2003. Intracellular survival of *Brucella*: defining the link with persistence. Vet Microbiol, 92: 213-223.

Geiss-Friedlander R and F Melchior, 2007.Concepts in sumoylation: a decade on.Nat Rev Mol Cell Biol, 8: 947-956.

Godfroid J,A Cloeckaert, JP Liautard, S Kohler, D Fretin, K Walravens, B Garin-Bastuji and JJ Letesson, 2005. 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, 36: 313-326.

Golding B, DE Scott, O Scharf, LY Huang, M Zaitseva, C Lapham, N Eller and H Golding, 2001. Immunity and protection against *Brucella abortus*. Microbes Infect, 3: 43-48.

Gross A, A Terraza, S Ouahrani-Bettache, JP Liautard and J Dornand, 2000. In vitro *Brucella suis* infection prevents the programmed cell death of human monocytic cells. Infect Immun, 68: 342-351.

Gul ST, A Khan, M Ahmad and I Hussain, 2013. Seroprevalence of brucellosis and associated hemato-biochemical changes in Pakistani horses. Pak J Agric Sci, 50: 447-452

Hamdy ME, SM El-Gibaly and AM Montasser, 2002. Comparison between immune responses and resistance induced in BALB/c mice vaccinated with RB51 and Rev.1 vaccines and challenged with *Brucella melitensis* bv. 3. Vet Microbiol, 88: 85-94.

Iqbal Z, H Jamil, ZI Qureshi, M Saqib, LA Lodhi, MS Waqas and M Safdar, 2013. Seroprevalence of ovine brucellosis by modified Rose Bengal test and ELISA in Southern Punjab, Pakistan. Pak Vet J, 33: 455-457.

Lacerda TL, PG Cardoso, L Augusto de Almeida, IL Camargo, DA Afonso, CC Trant, GC Macedo, E Campos, SL Cravero, SP Salcedo, JP Gorvel and SC Oliveira, 2010. Inactivation of formyltransferase (wbkC) gene generates a *Brucellaabortus* rough strain that is attenuated in macrophages and in mice.Vaccine, 28: 5627-5634.

Matunis MJ, E Coutavas and G Blobel, 1996.A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex.J Cell Biol, 135: 1457-1470.

Mauri F, LM McNamee, A Lunardi, F Chiacchiera, G Del Sal, MH Brodsky and L Collavin, 2008. Modification of Drosophila p53 by SUMO modulates its transactivation and pro-apoptotic functions. J Biol Chem, 283: 20848-20856.

O'Callaghan D, C Cazevieille, A Allardet-Servent, ML Boschiroli, G Bourg, V Foulongne, P Frutos, Y Kulakov and M Ramuz, 1999. 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, 33:1210-1220.

Pardi N, E Vámos, Z Ujfaludi, O Komonyi, L Bodai and IM Boros, 2011.In vivo effects of abolishing the single canonical sumoylation site in the C-terminal region of Drosophila p53. Acta Biol Hung, 62: 397-412.

Ribet D, M Hamon, E Gouin, MA Nahori, F Impens, H Neyret-Kahn, K Gevaert, J Vandekerckhove, A Dejean and P Cossart, 2010.Listeria monocytogenes impairs SUMOylation for efficient infection. Nature, 464: 1192-1195.

Sathiyaseelan J, R Goenka, M Parent, RM Benson, EA Murphy, DM Fernandes, AS Foulkes and CL Baldwin, 2006. Treatment of *Brucella* susceptible mice with IL-12 increases primary and secondary immunity. Cell Immunol, 243: 1-9.

Skendros P, G Pappas and P Boura, 2011. Cell-mediated immunity in human brucellosis. Microbes Infect, 13:134-142.

Smith M, W Turki-Judeh and AJ Courey, 2012. SUMOylation in *Drosophila* Development. Biomolecules, 2: 331-349.

Spallek T, S Robatzek and V Göhre, 2009. How microbes utilize host ubiquitination. Cell Microbiol, 11:1425-1434.

Stulemeijer IJ and MH Joosten, 2008.Post-translational modification of host proteins in pathogen-triggered defence signalling in plants. Mol Plant Pathol, 9: 545-560.

Sun D, P Xu and B He, 2011.Sumoylation of the P protein at K254 plays an important role in growth of parainfluenza virus 5. J Virol, 85: 10261-10268.

Vrioni G, G Pappas, E Priavali, C Gartzonika and S Levidiotou, 2008.An eternal microbe: *Brucella* DNA load persists for years after clinical cure. Clin Infect Dis, 46:e131-136.