Immune response and differentially expressed proteins in the lung tissue of BALB/c mice challenged by aerosolized Brucella melitensis 5

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Abstract

Objective: This study was performed to develop a murine aerosol infection model of brucellosis to investigate the pathogenicity and immune reactions induced by aerosolized Brucella and to identify key proteins associated with Brucella infection in lung tissue.

Methods: BALB/c mice were exposed to aerosolized Brucella melitensis 5 (M5) for 30 minutes and killed at 1, 3, 7, and 15 days post-exposure. Clinical observation, pathological analysis of lung tissue, and cytokine expression detection were then performed. Proteomic analysis based on two-dimensional electrophoresis and mass spectrometry was used to identify proteins exhibiting significant changes in expression in lung tissues during Brucella infection.

Results: Pathological analysis revealed alveolar wall thickening, telangiectasia with hyperemia, inflammatory cell infiltration, large areas of congestion and bleeding, and areas of focal necrosis. The T-helper 1 type immune response played an important role during aerosol infection, and 12 differentially expressed proteins were involved in the infectious process in lung tissue.

Conclusion: These results contribute to our understanding of the pathogenic process of Brucella in the lung tissue of BALB/c mice challenged with aerosolized Brucella. Some of the identified proteins may be potential targets in future therapeutic strategies.

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**Introduction**

Brucellosis, a zoonosis that affects several domestic animal species, manifests in humans as a systemic and febrile illness.\(^1\) *Brucella* is transmitted through ingestion, direct contact, or inhalation. Most patients develop this disease as the result of occupational exposure to animals or through the ingestion of unpasteurized dairy products\(^2\) or contaminated animal products from disease-endemic areas.\(^3\) Brucellosis is the most common zoonotic infection worldwide; more than 500,000 new infections are reported annually.\(^4\) Consequently, *Brucella melitensis*, *B. abortus*, and *B. suis* have been classified as category B agents by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases in the United States.\(^5\) There is a risk that these species may also be used as bioweapons.\(^6\)

*Brucella* causes infection after inhalation or invasion through cuts in the skin. Given the transmissibility of *Brucella* in an aerosolized form, laboratory workers are at high risk when handling contaminated specimens.\(^7,8\) In 2004, the CDC reported two *B. melitensis* infections related to epidemiological laboratories in a 57-year-old female laboratory worker (2001) and a 48-year-old woman (2002).\(^9\) In 2004, Mense et al.\(^10\) infected rhesus macaques with *B. melitensis* strain 16M via aerosol exposure and observed pathologies in the infected rhesus macaques similar to those observed in humans with brucellosis. In 2007, Kahl-McDonagh et al.\(^4\) used aerosolized *Brucella* to perform a long-term follow-up of bacterial survival in the lungs of mice.

*Brucella* is a facultative and intracellular bacterial pathogen, and the pathogenesis of brucellosis and nature of the protective immune response are closely related to these characteristics.\(^11\) Unlike other pathogens, *Brucella* does not encode classical pathogenic factors that directly harm eukaryotic cells.\(^12\) Instead, tissue damage results from indirect mechanisms, most likely through the activation of host immune responses after *Brucella*-derived antigens are recognized by immune receptors.\(^13\) Previous studies have revealed histological changes in mice infected with *Brucella* through the respiratory route, and the early lung immune response to *Brucella* has also been investigated.\(^14–16\) In 2001, however, Mense et al.\(^14\) observed no histologic changes in the lungs of inoculated mice; only changes in the spleen and liver became more severe as the dose and time following intranasal inoculation with *Brucella melitensis* strain 16M increased. In 2017, Hielpo et al.\(^15\) observed a mild proinflammatory response in murine lungs infected with *B. abortus* because of the immune modulation by its Btp proteins, which might facilitate its survival and dissemination to peripheral organs. In 2016, Hanot Mambres et al.\(^16\) explored the role of different pulmonary immune effectors in the protection against aerosolized *B. melitensis*. Their findings demonstrated that the nature of the protective memory response depends closely on the route of
infection and highlighted the role of interferon (IFN)-γ- and interleukin (IL)-17RA-mediated responses in the control of mucosal infection by Brucella. However, few studies to date have been performed to investigate the mechanisms of Brucella-induced cell damage.

This study was designed to establish a murine model of brucellosis using an aerosol exposure method and to investigate the pathogenicity and immune reactions induced by Brucella in lung tissue. Furthermore, we attempted to identify the key proteins involved in Brucella infection and characterize their biological functions. The results in this study may provide a foundation and useful baseline data for future studies of Brucella pathogenesis and vaccine development.

**Materials and methods**

**Animals**

Six-week-old female BALB/c mice (weight range, 20–22 g) were purchased from the Experimental Animal Centre, Liaoning, China and housed in specific pathogen-free conditions at the Experimental Animal Centre. Ten animals were used in each group. The animals were housed using a 12-hour light/12-hour dark cycle with free access to food and water. All animal studies were conducted in strict accordance with the guidelines for animal welfare of the World Organization for Animal Health. Experimental protocols involving animals were approved by the Animal Care and Use Committee of Academy of Military Medical Sciences, Beijing, China (approval number: SCXK 2016-0008).

**Aerosol Brucella infection in mice**

*Brucella melitensis* 5 (M5) was provided by Prof. Xinglong Wang at our research institute. Brucella was grown in tryptic soy broth (Sigma-Aldrich, St. Louis, MO, USA) at 37°C.

Two doses of *Brucella* (1 × 10^6 and 1 × 10^9 CFU/mL) were added to a generator (3079A; TSI Incorporated, Shoreview, MN, USA) at a flow rate of 200 L/hour. An aerodynamic particle sizer (APS3321; TSI Incorporated) was used to monitor the Brucella aerosol particle size and concentration. The median particle size was 0.84 μm and the aerosol particle concentration was 1083/cm^3^. Experiments were performed at 25°C with 60% humidity. A 0.9% sodium chloride solution without Brucella was used for the control group. Mice were exposed for 30 min and then killed at various time points up to 15 days post-exposure. Infected lung tissues were aliquoted and maintained at −80°C for subsequent experiments. Because the lung tissue mass of mice is relatively small (0.1 g/mouse) and the respiratory tract infection in mice is uneven, the whole lung tissue was used for the subsequent experimental study of cytokines and other host proteins.

**Histology**

Lung tissues (left and right) were harvested from three female BALB/c mice in each group at 1, 3, 7, and 15 days post-exposure. The harvested tissues were then fixed with 10% neutral buffered formalin and paraffin-embedded before 4-μm-thick longitudinal sections were cut and stained with hematoxylin and eosin according to standard protocols.

**RNA isolation and real-time reverse transcription polymerase chain reaction**

Total RNA was extracted from infected lung tissues using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.
The extracted RNA was resuspended in 100 µL of diethyl pyrocarbonate water and stored at −80°C. A reverse transcription kit (Qiagen) was employed to synthesize complementary DNA.

Real-time polymerase chain reaction (PCR) was performed using an ABI 7500 Real-Time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). All samples were run in triplicate. β-Actin was used as a reference gene to quantify host DNA in the sample. All primers shown in Table 1 were designed with Primer Express Software (Applied Biosystems) according to technical parameters to achieve low levels of penalty coupling factors.

Protein sample preparation and two-dimensional electrophoresis

Lung tissue samples from the control group and aerosol-infected group (7 and 15 days post-infection) were ground using liquid nitrogen and suspended in lysis buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The suspensions were homogenized, sonicated on ice, and centrifuged at 14,000 rpm for 1 hour at 4°C. The supernatants were then collected and protein concentrations determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The protein aliquots (100 mg each) were stored at −80°C for future use. Two-dimensional electrophoresis (2-DE) (Bio-Rad Laboratories) was performed to separate proteins. The resulting 2-DE gels were scanned using a GS-710 imaging densitometer, and digitized images were analyzed with PDQuest software (Bio-Rad Laboratories).

Mass spectrometry

As described in a previous study,17 protein gel spots were cut, destained, and washed before being placed in 0.2 M NH₄HCO₃ for 20 minutes and lyophilized. Each spot was digested overnight with 12.5 ng/mL trypsin in 0.1 M NH₄HCO₃, and the digested proteins were extracted three times with a 50% acetonitrile/0.1% trifluoroacetic acid solution. All mass spectra data were acquired using matrix-assisted laser desorption/ionization–time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (AutoFlex mass spectrometer with LIFT technology; Bruker Daltonics, Bremen, Germany).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± standard deviation.

Results

Clinical observation

The mice were divided into two dose groups (1 × 10⁶ and 1 × 10⁹ CFU/mL) and a
control group. During the early stage of infection, particularly the first 3 days, the high-dose group exhibited a greater number of symptoms, including depression and loss of appetite, than did the low-dose group. As the post-exposure time increased, the mice in the two groups gradually began to exhibit similar symptoms before being killed at 15 days post-exposure. No death was observed in either dose group over the 15-day course of the experiment. The weights of the mice in the control group gradually increased, while the weights of the mice in the two challenged groups remained almost unchanged (Figure 1).

**Pathological analysis of lung tissue**

As shown in Figure 2, the lung tissues in the control group had normal structures and intact alveolar walls. Thickening of the alveolar wall and telangiectasia with hyperemia were observed in the left lungs of both the low-dose and high-dose infected groups at 1 day post-exposure. At 3 days post-exposure, mice from the low-dose group showed further thickening of the alveolar wall, while mice from the high-dose group showed local structural damage and a small amount of inflammatory cell infiltration, mainly lymphocytes and macrophages. At 7 days post-exposure, the low-dose group exhibited cavity atrophy, and the high-dose group showed severe tissue damage, including a large number of infiltrating inflammatory cells. At 15 days post-exposure, both groups demonstrated large areas of congestion and bleeding as well as areas of focal necrosis. The right lungs exhibited pathological changes similar to those in the left lungs, but the tissue damage appeared more severe.

**Cytokine expression**

Figure 3 shows that all T-helper 1 (Th1)-type cytokines [IL-2, IL-12, IFN-γ, and tumor necrosis factor-α (TNF-α)] were upregulated in the lungs of *Brucella*-challenged mice, and the expression levels were much higher in the high-dose than

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**Figure 1.** Weight changes of BALB/c mice exposed to *Brucella*. The weights of mice in the control group gradually increased, while the weights of the mice in the two challenged groups remained almost unchanged. High dose, $1 \times 10^9$ CFU/mL; low dose, $1 \times 10^6$ CFU/mL.
low-dose group (P < 0.05). TNF-α, IL-2, and IL-12 exhibited peak expression at 3 days post-exposure, and IFN-γ exhibited peak expression at 7 days post-exposure. In terms of Th2-type cytokines (IL-4 and IL-10), IL-4 was also up-regulated with peak expression at 3 days post-exposure. However, no significant changes in IL-10 expression were observed during the course of experimental infection. These results indicate that the Th1 immune responses of mice were obviously activated after exposure to Brucella aerosol.

2-DE and mass spectrometry
Proteomics has been widely applied to develop two-dimensional polyacrylamide gel electrophoresis maps and databases for evaluating gene expression profiles of bacterial pathogens. In the present study, related techniques were used to identify proteins exhibiting significant changes in expression within lung tissues during Brucella infection. These findings are important to understand Brucella infection initiated through the aerosol route.

As shown in Figure 4 and Table 2, protein spots were selected and identified by performing MALDI-TOF mass spectrometry. Thirteen proteins were successfully identified; 1 had the same composition, resulting in 12 unique proteins. Among these 12 proteins, apolipoprotein A-I, peroxiredoxin 6, albumin, Ywhae 14-3-3 protein, peroxiredoxin 2, and proteasome

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**Figure 2.** Histological analysis of lung tissue after challenge by aerosolized Brucella. BALB/c mice were challenged with Brucella (1 × 10⁶ and 1 × 10⁹ CFU/mL) and killed at 1, 3, 7, and 15 days post-exposure. Lung tissues were collected and histologically examined, and the findings were compared with those in the control group.
Figure 3. Kinetics of the expression of Th1/Th2 cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-10, and IL-12) in lung tissues of BALB/c mice with aerosol exposure to *Brucella* compared with that of control group. High dose, $1 \times 10^9$ CFU/mL; low dose, $1 \times 10^6$ CFU/mL. *P < 0.05, **P < 0.01. Th1, T-helper 1; Th2, T-helper 2; IFN, interferon; IL, interleukin.
activator subunit 1 were found to be up-regulated, while creatine kinase, selenium-binding protein1 (SBP1), vimentin, histone H2B type 1-M, tropomyosin 2, and polymerase I and transcript release factor (PTRF) were found to be down-regulated. The proteins demonstrating increased expression were associated with protein

### Table 2. List of the identified differentially expressed proteins.

| SSP  | Gene ID  | Name                                      | MW (kDa) | pI    |
|------|----------|-------------------------------------------|----------|-------|
| A. Up-regulated proteins in the infection group versus control group | | | | |
| 3004 | 11806    | Apolipoprotein A-I                         | 30.4     | 5.52  |
| 6001 | 11758    | Peroxiredoxin 6                           | 24.9     | 5.98  |
| 7509 | 11657    | Albumin                                   | 67.0     | 5.49  |
| 0104/1107 | 22627 | Ywhae 14-3-3 protein epsilon               | 29.3     | 4.63  |
| 2003 | 21672    | Peroxiredoxin 2                           | 21.9     | 5.2   |
| 5102 | 19186    | Proteasome (prosome, macropain) activator subunit 1 | 28.8     | 5.73  |
| B. Down-regulated proteins in the infection group versus control group | | | | |
| 8206 | 12715    | Creatine kinase                           | 43.2     | 6.58  |
| 6301 | 20341    | Selenium-binding protein 1                | 53.1     | 5.87  |
| 1301 | 22352    | Vimentin                                  | 53.7     | 5.06  |
| 1002 | 319186   | Histone H2B type 1-M                      | 13.9     | 10.31 |
| 1202 | 22004    | Tropomyosin 2 (beta)                      | 32.9     | 4.66  |
| 403  | 19285    | Polymerase I and transcript release factor | 43.9     | 5.43  |

SSP, standard spot; ID, identification; MW, molecular weight; pI, isoelectric point.
Proteins with score of 100 (i.e., 100% homology) were included. SSP numbers are numbers assigned to each protein spot by PDQuest software, and each SSP number uniquely identifies one protein.

**Figure 4.** Representative two-dimensional gel electrophoretograms of lung tissues from the control group and infected group. (a) Control group. (b) Seven days post-exposure to aerosolized *Brucella* (*1 × 10^7 CFU/mL*). (c) Fifteen days post-exposure to aerosolized *Brucella* (*1 × 10^7 CFU/mL*). (d) PDQuest software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyze the scanned two-dimensional electrophoresis gel image. The spots representing significantly different protein expression were selected (Table 2) for further mass spectrometric analysis. The red pane shows the differentially expressed proteins.
transportation, antioxidant function, and antiviral or cell activation. The proteins with decreased expression were associated with cytoskeletal structure, enzyme activation, or cell intoxication and transformation.

**Discussion**

*Brucella* is a Gram-negative, facultative, intracellular bacterium that causes zoonotic brucellosis in humans and various animals. Brucella can be transmitted via the aerosol route. We established an aerosol-ized *Brucella* mouse infection model for the present brucellosis inhalation study. The focus of this study was to investigate the pathogenicity and immune reactions induced by *Brucella* and identify differentially expressed proteins involved in the respiratory response to *Brucella* infection.

Histological analysis indicated obvious structural damage and inflammatory cell infiltration (mainly lymphocytes and macrophages). In 2003, Ko and Splitter reported the ability of *Brucella* to infect phagocytic macrophages *in vivo* and *in vitro*. The virulence of *Brucella* relies on bacterial survival and replication in the vacuolar phagocytic compartments of macrophages. Many *Brucella* virulence factors are critical for intracellular replication in macrophages, which typically leads to systemic inflammation and whole-body reactions. The results of a previous study suggest that *B. abortus* survives in alveolar macrophages after inhalatory infection in spite of a certain degree of immune control exerted by the Toll-like receptor 2 (TLR2)-mediated inflammatory response, which may also be enhanced by the modest nature of this inflammatory response and the modulation of major histocompatibility complex II expression by the bacterium.

In their histological study, Hielpos et al. found that mice infected for 2 days had no peribronchial or perivascular infiltrate and showed only a mild and focal-ized lymphocytic interstitial infiltrate and a few points of hematic extravasation. On the 7th day of infection, the interstitial infiltrate was milder and some hematic extravasation was observed. In a study by Mense et al., histological changes in the spleen and liver were observed in infected mice, but no histological changes in the lungs were observed. In the present study, the pathological changes in lung tissue after *Brucella* infection were observed with increasing post-infection time (1, 3, 7, and 15 days post-infection). Thickening of the alveolar wall, telangiectasia with hyperemia, local structural damage, inflammatory cell infiltration, and even severe tissue damage were found as the post-infection time increased.

The Th1 immune response reportedly plays an important role during *Brucella* infection through the aerosol exposure route. Infection with the live strain RB51 typically leads to a Th1-specific immune response characterized by the induction of specific CD8+ cytotoxic T cells and CD4+ helper T cells that secrete IFN-γ. In 1995, Zaitseva et al. reported that heat-killed *B. abortus* promotes a strong Th1-type immune response. Both antibody- and cell-mediated immune responses affect *Brucella* infection, but cell-mediated immune responses are essential for intracellular bacterial clearance.

Th1 cells are mainly involved in cellular immunity and delayed hypersensitive inflammatory reactions, while Th2 cells can assist B cells to differentiate into antibody-secreting cells and participate in humoral immunity. The factors secreted by Th1 cells mainly include IL-2, IFN-γ, and TNF-α, which can mediate the immune response related to cytotoxicity and local inflammation and assist in the production of antibodies. Th1 cells thus participate in the occurrence of cellular immunity and delayed hypersensitive
inflammatory reactions and play an extremely important role in the body’s resistance to intracellular bacteria and other pathogens. The acute inflammation associated with respiratory tract infection is accompanied by a large amount of pre-inflammatory factors, which are largely diversified and balanced with one another. Only in this way can granulocyte aggregation in the alveolar cavity be stimulated and the antimicrobial mechanism of the body be activated.

In this study, 12 proteins were significantly differently expressed in Brucella-challenged murine lung tissues. The biological functions of these proteins are summarized as follows.

SBP1 is a cell malignancy biomarker and is important for maintaining normal cellular functions. According to the results of a microarray analysis conducted by Eskra et al., SBP1 expression dropped by 4.7-fold in RAW264.7 macrophages infected with B. abortus. Our results also showed changes in SBP1 expression during Brucella infection, suggesting that this protein may provide meaningful information regarding the mechanisms of aerosolized Brucella infection.

Vimentin, tropomyosin 2, and histone H2B type 1-M are cytoskeletal proteins that play critical roles in diverse physiological processes such as cell morphological change, motility, and migration. In a previous study, infection with the intracellular bacterium Mycobacterium tuberculosis induced the up-regulation of vimentin expression in human monocytes. In the present study, all three of the above-mentioned proteins were down-regulated. Although M. tuberculosis and B. melitensis are intracellular pathogens adapted to life inside macrophages, they have different structures and pathogeneses. The details of the vimentin-associated cellular mechanisms involved in the responses to these two bacteria need further exploration.

Peroxiredoxins 2 and 6 were up-regulated in this experiment. This is consistent with the findings reported by Rossetti et al., who assessed the host and pathogen transcriptional profiles of acute B. melitensis infection. Peroxiredoxin 6 is always highly expressed in type II pneumocytes and is involved in the lung injury induced by aerosolized Brucella.

Ywhae 14-3-3 proteins were originally discovered as a family of proteins that had high expression in the brain. Through their interactions with other binding partners, 14-3-3 proteins can influence many brain functions including neural signaling, neuronal development, and neuroprotection. The present study is the first to show that Ywhae 14-3-3 proteins participate in Brucella inhalation infection. The reason for the up-regulation of Ywhae 14-3-3 proteins requires further investigation.

PTRF is suggested to be a caveolar coat protein that controls and stabilizes both caveolar structure and function. In 2013, Zheng et al. reported that PTRF deficiency led to lower inducible nitric oxide synthase (iNOS) and nitric oxide (NO)/reactive oxygen species production in macrophages in vitro and that PTRF was also a crucial regulator of TLR4 signaling in the development of sepsis. NO has also been shown to accelerate Brucella elimination and induce IFN-γ and other anti-Brucella antibodies. Therefore, its down-regulated expression in our aerosol exposure studies may strengthen the need for further exploration of the relationships between Brucella inhalation infection and NO/iNOS signaling pathways.

Additional studies are required to establish whether changes in the levels of these proteins are attributable to direct effects or are consequences of interactions with other targets. These results contribute to our understanding of the Brucella pathogenic process, particularly that initiated via the aerosol infection route. In previous studies,
only a mild proinflammatory response in murine lungs infected with *B. abortus* was found due to the immune modulation by its Btp proteins; pulmonary manifestations of airborne *Brucella* infection are very rare.\(^{15,35}\) Additionally, this special immune response in mouse lung tissue might help the bacteria to escape clearance by the immune system. In this study, we explored the differentially expressed proteins in mouse lung tissue infected with *Brucella* and found that these proteins might contribute to the *Brucella* survival and even dissemination to peripheral organs. Further studies are needed to explore the interactions between these differentially expressed proteins and bacterial proteins, which may help us to determine why *Brucella* disseminates very rapidly from the lungs to peripheral organs, hampering therapeutic interventions.

**Declaration of conflicting interest**
The authors declare that there is no conflict of interest.

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