Identification of Brucella abortus virulence proteins that modulate the host immune response

Yufei Wang,1,4* Zeliang Chen,1,4* Yefeng Qiu,2† Yuehua Ke,1 Jie Xu,1,3 Xitong Yuan,1 Xianbo Li,1,4 Simei Fu,1,3 Mingquan Cui,1,4 Yongfen Xie,1 Xinying Du,1 Zhonjia Wang and Liuyu Huang1

1Department of Infectious Disease Control; Institute of Disease Control and Prevention; Academy of Military Medical Science; Beijing, China; 2Experimental Animal Center; Academy of Military Medical Science; Beijing, China; 3School of Public Health; Key Laboratory of Zoonosis; Ministry of Education; Institute of Zoonosis; Jilin University; Changchun, China; 4College of Veterinary Medicine; Sichuan Agricultural University; Ya’an, China

†These authors contributed equally to this work.

Brucellosis is an important zoonotic disease of almost worldwide distribution. One significant immune phenomenon of this disease is the ability of the pathogen to hide and survive in the host, establishing long-lasting chronic infections. Brucella was found to have the ability to actively modulate the host immune response in order to establish chronic infections, but the mechanism by which the pathogen achieves this remains largely unknown. In our screening for protective antigens of Brucella abortus, three proteins (BAB1_0597, BAB1_0917 and BAB2_0431) were found to induce significantly higher levels of gamma interferon (IFNγ) in splenocytes of PBS immunized mice than those immunized with S19. This finding strongly implied that these three proteins inhibit the production of IFNγ. Previous studies have shown that LPS, PrpA and Btp1/TcpB are three important immunomodulatory molecules with the capacity to interfere with host immune response. They have been shown to have the ability to inhibit the secretion of IFNγ, or to increase the production of IL-10. Due to the role of these proteins in virulence and immunomodulation, they likely offer significant potential as live, attenuated Brucella vaccine candidates. Understanding the mechanisms by which these proteins modulate the host immune responses will deepen our knowledge of Brucella virulence and provide important information on the development of new vaccines against Brucellosis.

Brucella spp is a Gram-negative, facultative, intracellular bacterium that causes abortion in domestic animals and undulant fever, endocarditis, arthritis and osteomyelitis in humans. Immunity against Brucellae requires cell-mediated mechanisms, in particular a T helper 1 (Th 1) immune response characterized by the production of gamma interferon (IFNγ), which is associated with protective immunity. Therefore, proteins which present T-cell epitopes to the host could be protective antigen candidates. Previous work in our laboratory led to the identification of Brucella protective antigens, proteins associated with Brucella pathogenesis were expressed in E. coli and their abilities to induce T-cell responses were tested. The purified proteins were used to stimulate the splenocytes of S19 or PBS (negative control) immunized mice and IFNγ secretions were quantified. Only those proteins that stimulate significantly higher levels of IFNγ in S19 immunized mice than those immunized with PBS were considered to induce cellular immune responses. Fortunately, a number of proteins were found to stimulate stronger IFNγ responses. These proteins were used to immunize BALB/c mice and the protective efficacies against virulent B. abortus infection were assessed. At last, two proteins were found to induce protective immune responses in mice.

Unexpectedly, we also found that three proteins (BAB1_0597, BAB1_0917 and BAB2_0431) induced significantly higher
levels of IFNγ in splenocytes of PBS immunized mice than those of S19 immunized mice. This very interesting phenomenon which was not observed in our previous studies on *Yersinia pestis*, where all tested proteins induced higher or identical levels of IFNγ in a vaccine strain immunized mice than control mice.\(^7\) We assumed that this phenomenon might be the result of interference of these proteins with the host immune responses; prompting us to carefully analyze the ability of Brucella to interfere with the host immune responses.

As an intracellular bacterial pathogen, the virulence of Brucella depends on its survival and replication properties within host cells. One significant immune phenomenon observed during brucellosis is the ability of the pathogen to hide and survive in the host, establishing long-lasting chronic infections.\(^8\) In general, microbial pathogens with the ability to establish chronic infections have evolved strategies to actively modulate the host immune response. Indeed, significant evidence exists to suggest that Brucella has the ability to avoid and/or interfere with host innate and acquired immune responses.\(^9\)

One strategy used by Brucella to subvert the host innate immune system is via modification of pathogen-associated molecular LPS. Unlike enterobacterial LPS, *B. abortus* lipid A contains a much longer fatty acid residue C28 other than C12–C16, and this modification greatly reduces and delays inflammatory response in the infected hosts compared with the endotoxins from other Gram-negative bacteria.\(^4\) In addition, due to the particular function and structure of Brucella LPS, which avoids the activation of the macrophage-killing systems and confers resistance to the pathogen against the microbicidal action of antibiotics, Brucella are able to survive and multiply inside phagocytic cells without provoking their apoptosis.\(^9\) Furthermore, Brucella LPS was described in vitro as a downregulator of CD4 T cell activation.\(^10\) Taken together, all these studies indicate that Brucella LPS plays a central role in the immunosuppression observed upon Brucella infection.

Another strategy used by Brucella to thwart an effective immune response might be to induce expansion of interleukin-1β-producing B cells.\(^11\) In the early stages of acute human brucellosis the predominant response is Th1, with IFNγ production by T cells and natural killer (NK) cells.\(^12\) However, in chronic human brucellosis, the cell-mediated immunity, mainly Th1, is transiently immunosuppressed in comparison to the antibody response.\(^5,12,13\) Previous studies have shown that *B. abortus* also induces the anti-inflammatory cytokine interleukin-10 (IL-10) in addition to an early Th1 response.\(^14,15\) IL-10 can inhibit the microbicidal activity of macrophages against Brucella, as well as antagonizing the activity of IFNγ.\(^16\) A *B. abortus* proline racemase, PrpA, was shown to induce a T cell-independent B cell nonspecific polyclonal activation concomitant with the secretion of IL-10.\(^16\) B cell polyclonal activation induced by different virus, bacteria and parasites has been strongly associated with immune suppression and PrpA is a T-independent B cell mitogen first identified in Brucella. The protein plays an important role in the modulation of the host immune response during Brucella infection. Whereas the wild type (WT) and the complemented *prpA* mutant strains induce a tempo- ral restricted unre sponsive status in the mouse, the *prpA* mutant is unable to achieve this.\(^16\)

Besides, several studies have shown a Brucella protein called Btp1 (also known as TcpB), which bears significant homology with the Toll/Interleukin-1 receptor (TIR) domain present in Toll-like receptors (TLRs) and adaptor molecules, also has immunomodulatory properties.\(^17,18\) TLRs play essential roles in the activation of innate immune responses against microbial infections. Btp1/TcpB has been shown to inhibit TLR2 and TLR4 mediated NF-κB activation.\(^19\) Therefore, Brucella could subvert TLR signaling pathways to suppress host immune responses to benefit their survival and persistence.

From the above discussion, we can conclude that LPS, PrpA and Btp1/TcpB, which could interfere with host immune responses, are able to inhibit the secretion of IFNγ, similarly with the three proteins (*BAB1_0597, BAB1_0917* and *BAB2_0431*) found in our study. Our results suggest *BAB1_0597, BAB1_0917* and *BAB2_0431* may have the ability to interfere with host immune responses. But the definite role of these virulence proteins in immune response regulation needs to be further experimentally verified.

Due to the role of these proteins in virulence and immunomodulation, they might have significant potential as live, attenuated Brucella vaccine candidates. To date, development of live, attenuated Brucella vaccines that are safe for use in humans has focused on the deletion of important genes which could interfere with host immune responses, are able to inhibit the secretion of IFNγ, similarly with the three proteins (*BAB1_0597, BAB1_0917* and *BAB2_0431*) found in our study. Our results suggest *BAB1_0597, BAB1_0917* and *BAB2_0431* may have the ability to interfere with host immune responses. But the definite role of these virulence proteins in immune response regulation needs to be further experimentally verified.

| Locus | Protein name | The location of Protein | Description |
|-------|--------------|-------------------------|-------------|
| BAB1_0597 | - | Unknown | Hypothetical cytosolic protein |
| BAB1_0917 | Tig | Cytoplasmic | Trigger factor |
| BAB2_0431 | - | Cytoplasmic | D-galacturonate dehydratase |

| Locus | Protein name | The location of Protein | Description |
|-------|--------------|-------------------------|-------------|
| BAB1_0597 | - | Unknown | Hypothetical cytosolic protein |
| BAB1_0917 | Tig | Cytoplasmic | Trigger factor |
| BAB2_0431 | - | Cytoplasmic | D-galacturonate dehydratase |

*Table 1. Proteins that inhibited the secretion of IFNγ in mice immunized with S19*
live attenuated vaccine candidates needs to be further verified. Understanding the mechanisms by which these three proteins modulate the host immune responses will help us to develop efficient vaccines against Brucellosis.

In conclusion, Brucella is an intracellular bacterial pathogen with the capacity to establish a chronic infection. In the past 10 years, the study of Brucella pathogenicity has been focused mainly on identifying factors that affect the intracellular trafficking and multiplication of the bacterium in the host cell. However, little is known about the molecular factors associated with chronicity and immunomodulation of the host. Our findings provide important clues in this regard. Understanding these mechanisms can be useful not only for the study of host immune regulation by Brucella, but also for the development of new vaccines or therapeutic agents against Brucellosis.

References
1. Young HJ. An overview of human brucellosis. Clin Infect Dis 1995; 21:285-9. PMID:9502733; http://dx.doi.org/10.1093/clinids/21.2.285.
2. Eze MY, Lyon K, Crawford RM, Panaretou CM, Hadfield TL, Blumencranz AH, et al. Effects of osteopontin and gamma interferon on growth of Brucella melitensis 19F in mouse peritoneal macrophages in vivo. Infect Immun 2000; 68:297-30.
PMID:10403366; http://dx.doi.org/10.1128/IAI.68.1.257-63.2000.
3. Fu Y, Xu L, Li X, Xin Y, Qiu Y, Du X, et al. Immunisation of mice with membrane protein CobA of Salmonella protects against Brucella infection. J Gen Virol 2011; 92:2185-93.
PMID:22338959; http://dx.doi.org/10.1128/JVI.05579-10.
4. Li B, Zhou L, Gao J, Wang X, Ni B, Ke Y, et al. High-throughput identification of new protective antigens from a live vaccine live strain by enzyme-linked immunospot assay. Infect Immun 2009; 77:4356-61; PMID:19651863; http://dx.doi.org/10.1128/IAI.00242-09.

4. 7.
6.
5.
14.
12.
10.
9.
8.
7.
6.
5.
4.
3.
2.
1.

8. Basques-Cabero E, Comal-Abalos R, Chacin-Diaz C, Quesada-Lobo L, Martinez-A, Grañés-Varela C, et al. The differential interaction of Brucella and Escherichia coli with mouse macrophages involves actin-dependent endocytosis, which is more effective in Brucella than in E. coli. Infect Immun 2010; 78:4449-58.
PMID:20045188; http://dx.doi.org/10.1128/IAI.00242-09.
9. Martinez de Tijeda G, Piart-Castillo I, Moriyon I, Moriyon J. The same membrane of Brucella spp. varies innate immunity results main related to the evolution of pathogenicity. PLoS One 2015; 10:e0137097; http://dx.doi.org/10.1371/journal.pone.0137097.
10. Fourt E, Moriyon E, Moriyon J, Piart-Castillo I, Weizmann A, Govers JF. Brucella-Tobamovirus chimeric chimaeras are less prone to hydrophobic probes and more sensitive to classical prokaryotes and E. coli than are their native Brucella up components. J Bacteriol 1994; 174:5467-76.
PMID:8381480.
11. Fontana C, Drioli F, Luppa N, Moroni E, Govers JF. Brucella abortus lipopolysaccharide in murine peritoneal macrophages acts as a downregulator of T cell activation J Immunol 2000; 165:5282-10.
PMID:10846095.
12. Giannakoukou GE, Delivizis MV, Cahainovitch ME, Watch AC, Balls PL, Vlietkowska CA, et al. Diminished production of T helper 1 cytokines correlates with the chronic disease phenotype in Brucella abortus-infected mice. J Immunol 2005; 175:4131-41.
PMID:16049449.
13. Elbak M, Al-Hokail AA. Transforming growth factor-beta production correlates with decreased interferon-gamma in humans with chronic brucellosis Microbes Infect 2008; 10:1889-96.
PMID:18405956; http://dx.doi.org/10.1016/j.micinf.2008.01.010.
14. Fernandez DM, Rubben CL. Identification of down-regulates protective immunity to Brucella abortus. Infect Immun 1995; 63:1530-3.
PMID:7484248.