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Evaluation of Th1/Th2-Related Immune Response against Recombinant Proteins of *Brucella abortus* Infection in Mice

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Brucellosis is a zoonotic disease caused by *Brucella*, a genus of gram-negative bacteria. Cytokines have key roles in the activation of innate and acquired immunities. Despite several research attempts to reveal the immune responses, the mechanism of *Brucella* infection remains unclear. Therefore, immune responses were analyzed in mice immunized with nine recombinant proteins. Cytokine production profiles were analyzed in the RAW 264.7 cells and naive splenocytes after stimulation with three recombinant proteins, metal-dependent hydrolase (r0628), bacterioferritin (rBfr), and thiamine transporter substrate-binding protein (rTbpA). Immune responses were analyzed by ELISA and ELISpot assay after immunization with proteins in mice. The production levels of NO, TNF-α, and IL-6 were time-dependently increased after having been stimulated with proteins in the RAW 264.7 cells. In naive splenocytes, the production of IFN-γ and IL-2 was increased after stimulation with the proteins. It was concluded that two recombinant proteins, r0628 and rTbpA, showed strong immunogenicity that was induced with Th1-related cytokines IFN-γ, IL-2, and TNF-α more than Th2-related cytokines IL-6, IL-4, and IL-5 in vitro. Conversely, a humoral immune response was activated by increasing the number of antigen-secreting cells specifically. Furthermore, these could be candidate diagnosis antigens for better understanding of brucellosis.

Keywords: *Brucella abortus*, cytokine, immunogenicity, recombinant protein

Introduction

Brucellosis is one of the major zoonotic diseases caused by facultative intracellular bacteria of genus *Brucella*. It also leads to huge economic losses for the livestock industry. The genus consists of 11 classical species in accordance to the primary preferred host and antigenic variation: *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats), *B. ceti* (seals), *B. pinnipedialis* (pinnipeds), *B. inopinata* (breast implant infection), and *B. papionis* (baboon) [12, 21, 29, 33]. *Brucella* spp. are able to establish a chronic infection in the host cells through evasion of the host bactericidal phagocyte functions and proliferate within macrophages [9, 10].

Infection of *B. abortus* activates the immune system and shows different clinical signs. This zoonotic pathogen causes undulant fever, endocarditis, arthritis, and osteomyelitis in humans, and abortion and infertility in cattle [13]. *B. abortus* remains in the reproductive tissue, fetal fluids, and the udders of animals. Therefore, it is easily able to infect between animals via ingestion or inhalation of the infected organisms. In humans, brucellosis is usually transmitted by direct contact or consumption of unpasteurized dairy products from infected animals. It is important to prevent and control both human and bovine brucellosis. It is important to focus on prevention of the spread of this disease using effective diagnostic methods and vaccines.

The lipopolysaccharide (LPS) has been considered to be the most important antigen during an immune response in brucellosis [2]. Moreover, a diagnosis of *B. abortus* in ruminant is mainly based on serological confirmation with LPS-based antigens. Most widely used are indirect diagnositc

approaches of brucellosis using serological tests, including standard tube agglutination test, Rose Bengal plate agglutination test, complement fixation test, and enzyme-linked immunosorbent assay (ELISA). Although LPS elicits a strong immune response, there are several problems using LPS-based diagnostic methods. LPS-based serological tests cannot easily differentiate between the infected and vaccinated animals [28]. Brucella O antigen is similar to various gram-negative bacteria, like *Yersinia enterocolitica* O:9 [20], *Escherichia coli* [27], and *Salmonella urbana* [25]. LPS based on the tests give false positive results due to these bacteria. Therefore, many researchers have tried to develop diagnostic reagents without LPS [1].

Recently, immunoproteomics have been investigated to identify specific immunogenic proteins of *Brucella*. Many research groups have identified several proteins as immune-reactive proteins in proteomic analysis of *B. abortus* [1, 7, 15, 22, 24]. Outer membrane proteins (Omps) have been considered as major immunoreactive components in the bacterial cells to develop new diagnostic or vaccine candidates [5, 6, 14, 30]. Many researchers have studied other recombinant proteins of *Brucella* to develop new diagnostic marker of brucellosis. Moreover, immunodominant antigens were investigated after immunization with *Brucella* spp. [23, 31]. Although many immunogenic antigens have been investigated, only a few mechanisms are known in brucellosis. Most microorganisms have developed a transport and storage system to enhance their survivability. Iron is one of the essential elements for their mechanisms. Bacterioferritin (Bfr) had been studied to be responsible for iron metabolism and survival in macrophages [8]. Additionally, Lee *et al*. [23] reported that metal dependent hydrolase (0628) and thiamine transporter substrate-binding protein (TbpA) were correlated with the pathogenesis and metabolism of *Brucella* infection, respectively. In this study, three proteins (0628, Bfr, and TbpA) were selected to analyze the possibility of LPS-free protein antigens in the diagnosis of bovine brucellosis through the investigation of immune responses of the recombinant proteins via in vitro tests.

Materials and Methods

Bacterial Strains and Growth Condition

The bacterial strains used in this study were *Brucella abortus* 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain, and *E. coli* DH5α (Invitrogen, USA). *B abortus* was cultured in *Brucella* broth (BD Bioscience, USA), overnight at 37°C in a gyratory shaker at 220 rpm. *E. coli* DH5α, producing the necessary plasmid constructs, was routinely grown at 37°C in Luria-Bertani (LB) broth (Duchefa, The Netherland) or agar supplemented with ampicillin (Sigma, USA). When solid medium and ampicillin were required, the above media were supplemented with 1.5% (w/v) agar (Takara, Japan) and 100 μg/ml of ampicillin (Sigma, USA).

Construction of 0628, Bfr, and TbpA Expression Clones

Total genomic DNA was prepared from *Brucella abortus* 544 culture using a G-spin Genomic DNA Extraction kit for bacteria (Intron, Korea). Genes encoding 0628, Bfr, and TbpA of *B. abortus* were amplified by PCR with the following primers pairs: 0628 sense primer 5′-AGCGCGGATCCATGCATTGTAAGATTCTG-3′, 0628 antisense primer 5′-AGCGCTGCAGTTAAGCTTGGAAGCTGTG-3′, Bfr sense primer 5′-AGCGGATCCATGAAAGGCGAACCAAAG GTC-3′, Bfr antisense primer 5′-ATCCTGCAGTTACTCAGCTTC GTCGGCGGG-3′, TbpA sense primer 5′-ACGGGATCCATGCGA CTTTTATCCTTGCTT-3′, and TbpA antisense primer 5′-AATACT GCAGTCATCTGCTGGTGGCTGCCA-3′. The amplified DNA was digested with appropriate restriction enzymes and ligated into a pCold TF vector (Takara, Japan). The recombinant plasmids were then transformed into the *E. coli* DH5α host cell for expression. Conservation of the correct sequences of the insert in the expression vector was confirmed by nucleotide sequencing.

Expression and Purification of r0628, rBfr, and rTbpA Proteins

The expression and purification of recombinant proteins were modified in accordance with a previous study [31]. In brief, *E. coli* containing fusion plasmids were cultured overnight and 20 ml of the bacteria was inoculated with one liter of ampicillin containing LB broth at 37°C for 7 h. Then, isopropyl β-D-1-thiogalactopyranoside (Amresco, USA) was added to the final concentration of 0.3 mM and further incubated at 37°C for 2 h. The bacterial cells were harvested by centrifugation at 4,400 ×*g* for 20 min. The supernatant was discarded and resuspended in 40 ml of column buffer (20 mM Tris HCl, 8 M urea, 500 mM NaCl, 20 mM imidazole, and 1 mM β-mercaptoethanol, pH 8.0). The samples were then sonicated at 10,000 Hz in an ice-water bath and centrifuged at 4,400 ×*g* for 20 min to collect the supernatant. The supernatant was then loaded onto a His SpinTrap (GE Healthcare, UK) column in accordance with the manufacturer's instructions. The purified proteins were stored at –20°C.

SDS-PAGE and Western Blot Assay

SDS-PAGE and western blotting were performed with the recombinant proteins. In brief, the purified recombinant proteins were diluted with sample buffer and boiled for 10 min at 100°C. After electrophoresis, the samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron, Korea). Proteins resolved by SDS-PAGE were transferred to the nitrocellulose membrane (Invitrogen, USA) for 25 min using an iBlot transfer device (Invitrogen). Membranes were blocked by incubation in 5% skim milk (BD, USA) for 1 h at room temperature (RT) and washed three times with washing buffer (TBS); then, they were incubated with anti-histidine (1:2,000 dilution; AprilBio Co., Ltd,

Korea) for 3 h at RT. The membranes were washed three times for 10 min each time with the same buffer, and then incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000 dilution; Bethyl, USA) for 1 h at room temperature. After final washing, the proteins were visualized with an AP conjugate substrate kit (Bio-Rad, USA).

Cytokines and NO Measurement in a Murine Macrophage Cell Line, RAW 264. 7

A murine macrophage cell line, RAW 264.7, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (50 μg/ml), and streptomycin (50 μg/ml) at 37°C under 5% CO_2. After incubating the cells for 8 h in the 12-well plates containing 1×10^6 cells/ml, they were stimulated with 10 μg/ml of r0628, rBfr, and rTbpA. The activity of LPS contaminated in the recombinant proteins was inhibited by incubation with polymyxin B (10 μg/ml) for 30 min before stimulation of macrophage. *E. coli* LPS (1 μg/ml; Sigma, USA) was used as the positive control and DPBS (Gibco, USA) was used as the negative control. The culture supernatants were collected at 4, 8, and 24 h after stimulation. The amounts of TNF-α, IL-6, IL-1β, IL-12p70, and IFN-γ were measured using the enzyme-linked immunosorbent assay according to the manufacturer's instruction (eBioscience inc., USA). The production of NO was measured by measuring the nitrite accumulation with the Griess reaction. Briefly, 100 μl aliquots of the culture supernatants were incubated with the same volume as the solution containing 1% sulfanilamide (Sigma, USA) and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid. They were then incubated for 10 min at room temperature and the absorbance was measured at 540 nm. Nitrite concentrations in each well were calculated based on the standard curve generated with sodium nitrite.

Isolation of Splenocytes and Cytokines Measurement in Splenocytes of Naive Mice

For the measurement of IFN-γ, IL-2, IL-4, and IL-5, splenocytes (1×10^6 cells/ml) were isolated from healthy 5-week-old BALB/c female mice (Orient-Bio, Korea) and stimulated with 10 μg/ml of r0628, rBfr, and rTbpA. After 24 h, the amounts of IFN-γ, IL-2, IL-4, and IL-5 in the culture supernatants were measured using the ELISA according to the manufacturer's instruction (eBioscience Inc., USA). Concanavalin A (Sigma), and the medium were used as the positive and negative controls, respectively. All stimuli were pretreated with polymyxin B to avoid LPS contamination. All care and handling of animals were performed with the approval of Seoul National University Institutional Animal Care and Use Committees (IACUC) and the approval number is SNU-150302-2-1.

Measurement of Cells Secreting IgG, IFN-γ, and IL-4 in Mouse Splenocytes

The 6-week-old BALB/c female mice were immunized by the intra-peritoneal injection of 30 μg of the purified recombinant proteins, r0628, rBfr, and rTbpA mixed with complete Freund's Adjuvant (CFA, Sigma, USA) on day 0 and within incomplete Freund's Adjuvant (IFA, Sigma, USA) on day 14. Recombinant r0628-, rBfr-, and rTbpA-specific IgG memory B cells and IFN-γ- and IL-4-secreting T cells from the spleen of immunized mice were measured by the Enzyme-Linked ImmunoSpot (ELISpotBasic) assay kit according to the manufacturer's instruction (Mabtech AB, Sweden). For IgG memory B cells, 200 μl of r0628, rBfr, and rTbpA in PBS (50 μg/ml) was added to the ELIspot plates after pretreatment with 70% ethanol and coated by overnight incubation at 4°C. For IFN-γ- and IL-4-secreting T cells, 200 μl of monoclonal antibody against IFN-γ (AN18, 15 μg/ml) and IL-4 (11B11, 15 μg/ml) were added to the plates and coated as described above. After incubation, the plates were extensively washed with PBS five times and blocked with RPMI1640 with 10% FBS for 30 min at room temperature. Upon removal of the medium, splenocytes isolated from mice at 28 days after first immunization were added into the wells at 2×10^5 cells/well concentration. The plates were incubated at 37°C under 5% CO_2 for 24 h for IgG or 48 h for IFN-γ and IL-4. After removing the cells, 100 μl of biotinylated anti-IgG, IL-4, and IFN-γ antibodies in PBS containing 0.5% FBS (PBS-0.5% FBS) was added to each well. After incubation for 2 h at RT, the plates were washed and streptavidin-HRP in PBS-0.5% FBS was added and incubated for 1 h at RT. Antigen-secreting cells (ASC) were visualized upon addition of ready-to-use TMB substrate solution after washing the wells with PBS. The numbers of ASC were counted using Eli.Scan+ (A.EL.VIS, Germany). The production of antibody was determined by ELISA using purified recombinant proteins as coating antigens. Goat anti-mouse IgG (Bio-Rad, USA), IgG1 (Southernbiotech, USA), IgG2a (Southernbiotech, USA), and IgM (Jackson Immuno, USA) were used to detect IgG, IgG1, IgG2a, and IgM, respectively. All samples were in triplicates.

Statistics

Statistical significance (p-value) was calculated using the Student t test with the Statistical Package for Social Science software ver. 4.0 (MS, USA). Differences were considered to be significant at a value of $p < 0.05$. All experiments were repeated at least three times.

Results

Cloning, Expression, and Purification of Recombinant 0628, Bfr, and TbpA

Cloning of the 0628, Bfr, and TbpA genes in the pCold TF expression system led to the expression of TF fusion proteins, which were then purified by a histidine column. The SDS-PAGE profiles of purified r0628, rBfr, and rTbpA were approximately 77.13, 70.57, and 88.76 kDa in size, respectively (Fig. 1A). The identity of the expressed recombinant proteins, r0628, rBfr, and rTbpA, were confirmed in expected size by western boltting with anti-histidine antibody (Fig. 1B).

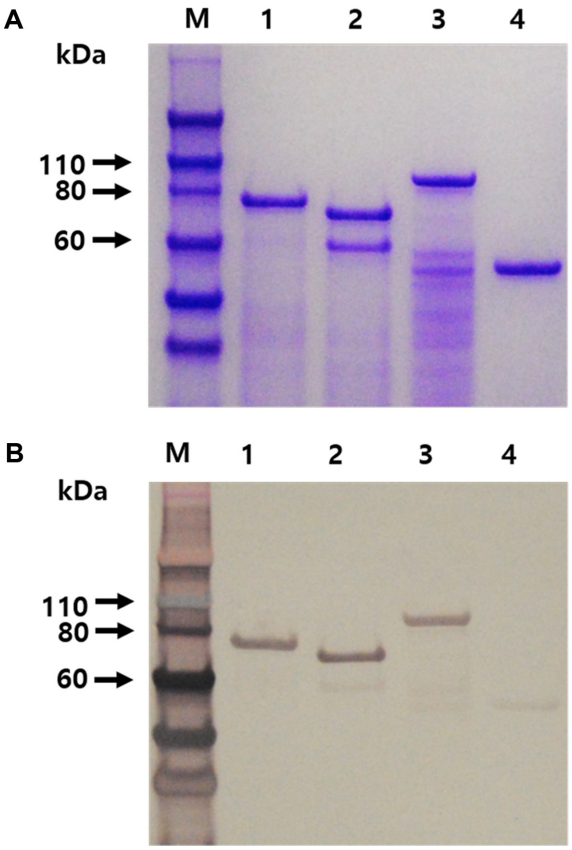

Fig. 1. Analysis of purified recombinant 0628, Bfr, and TbpA of *Brucella abortus*.

SDS-PAGE (**A**) and western blot (**B**) analysis of soluble protein fractions of pCold TF expressed with expected size. M: molecular weight markers; Lane 1: 0628 (77.13 kDa); Lane 2: Bfr (70.57 kDa); Lane3: TbpA (88.76 kDa); Lane 4: vehicle (52 kDa).

Production of TNF-α, IL-6, and NO in RAW 264.7 Cells

Culture supernatants of RAW 264.7 cells were assayed for TNF-α, IL-6, and NO production at 4, 8, and 24 h after stimulation with r0628, rBfr and rTbpA, by ELISA and Griess assay. The amounts of TNF-α from the stimulating groups with three recombinants proteins were higher than those from the group stimulated with DPBS ($p < 0.01$) (Fig. 2B). Similarly, a significant amount of IL-6 in the recombinant proteins exposure group was produced ($p < 0.01$) (Fig. 2C). The production of NO in the experimental groups was also significantly higher than the DPBS control group at 24 h after stimulation with the three recombinant proteins ($p < 0.01$) (Fig. 2A). However, other cytokines, IL-1β, IL-12p70, and IFN-γ, were not detected. Thus, the three recombinant proteins were proven to have immune-stimulating activities in RAW 264.7 cells by production of TNF-α, IL-6, and NO.

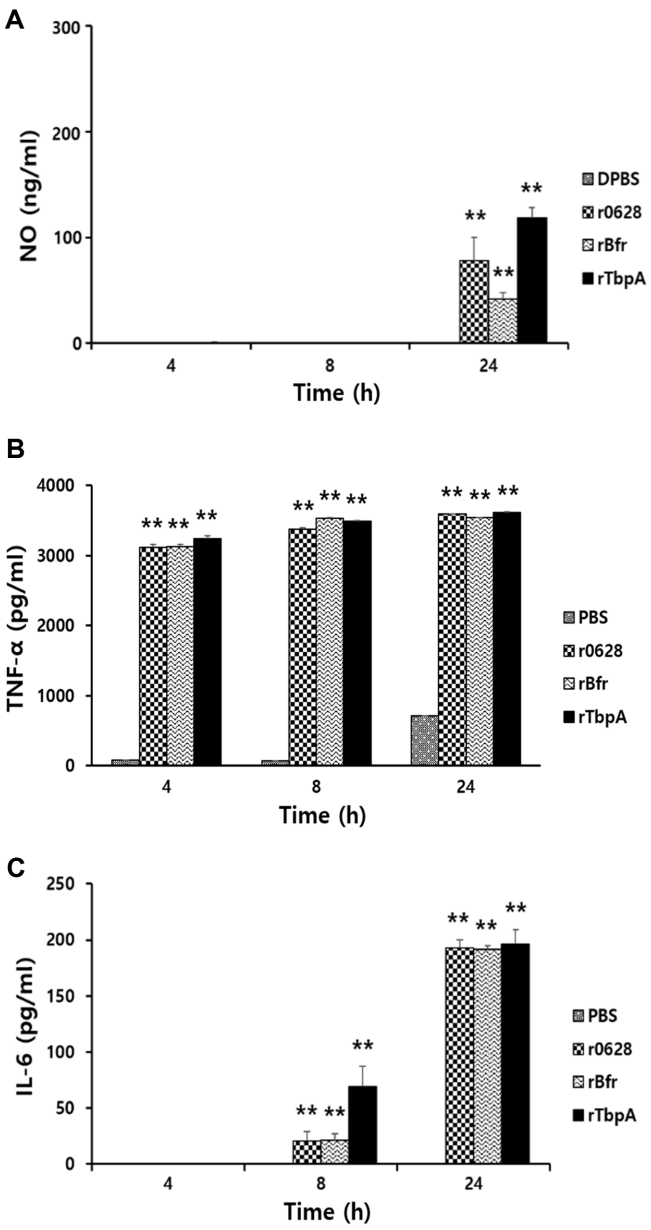

Fig. 2. Productions of nitric oxide (NO) and inflammatory cytokines in RAW 264.7 cells after stimulation with the recombinant proteins.

NO production showed at 24 h after stimulation with the proteins (**A**). Production of TNF-α was increased at 4 h (**B**), whereas IL-6 production was gradually increased after stimulation with the proteins (**C**).

Production of Cytokines in Naive Mouse Splenocytes

The amounts of IFN-γ and IL-2, marker cytokines for Th1 response, and IL-4 and IL-5, marker cytokines for Th2 response, in naive mouse splenocyte culture supernatants were measured at 12 and 24 h after stimulation with the three recombinant proteins by ELISA. The production of

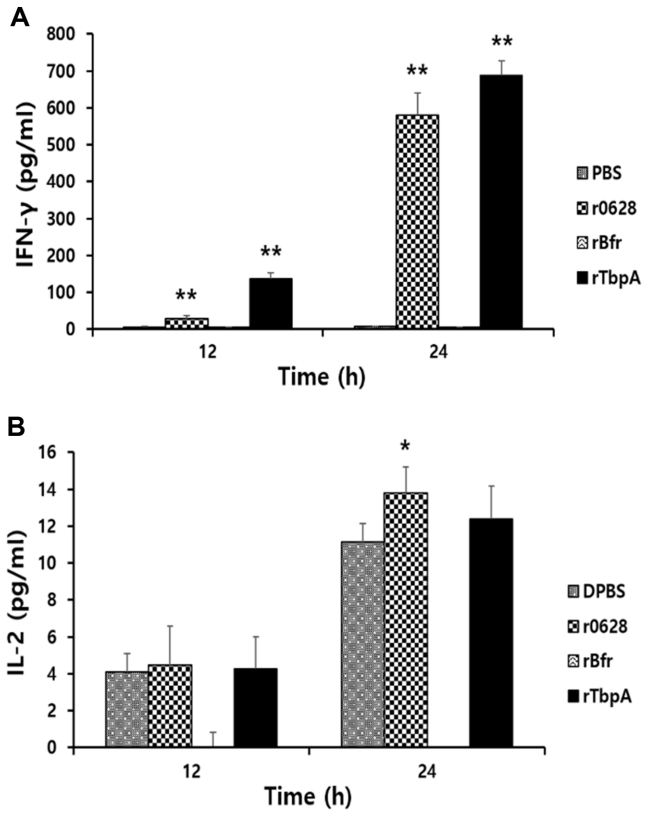

Fig. 3. Production level of cytokines from naive mouse splenocytes stimulated with *Brucella abortus* recombinant proteins.

Production of INF-γ (**A**) and IL-2 (**B**), Th1-related cytokines, was gradually increased in a time-dependent manner after stimulation with r0628 and rTbpA.

IFN-γ from the mouse splenocytes stimulated with r0628 and rTbpA was higher than that from the DPBS-stimulated group, in a time-dependent manner ($p < 0.01$) (Fig. 3A). The production of IL-2 was observed at 24 h in the r0628-stimulated group after stimulation ($p < 0.05$) (Fig. 3B). However, the production of IL-4 and IL-5, marker cytokines for Th2 response, from the mouse splenocytes stimulated with the three recombinant proteins did not show any difference, compared with the DPBS-stimulated group. In summary, r0628 and rTbpA, but not rBfr, have significant effects on inducing IFN-γ release from naive mouse splenocytes. On the contrary, there was no difference in IL-2 expression on the splenocytes before and after stimulation with the three recombinant proteins, as shown in Fig.3.

Analysis of Immune Cells in Splenocytes of Mice Immunized with r0628, rBfr, and rTbpA

The number of antigen-specific IgG-secreting B cells

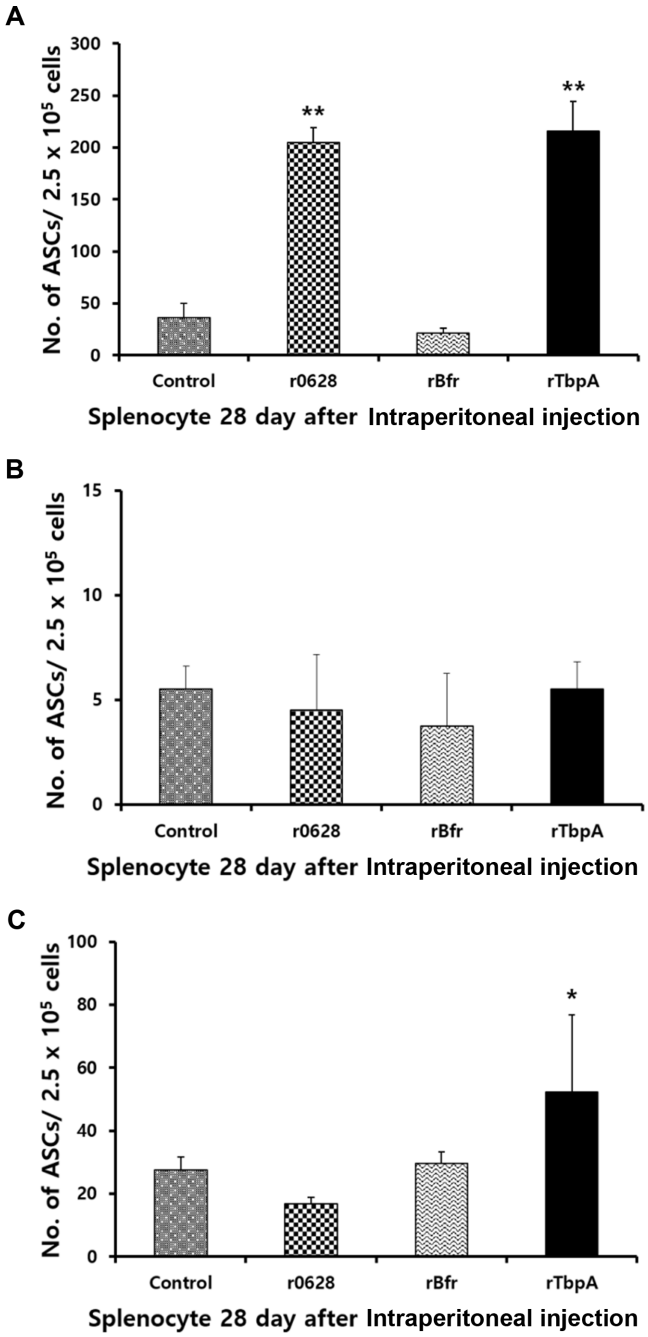

Fig. 4. Number of antigen-secreting cells (ASC) from splenocytes of mice immnunized with r0628, rBfr, and rTbpA of *Brucella abortus* at 28 days post immunization.

(**A**) Total IgG, (**B**) IFN-γ, and (**C**) IL-4.

from the mouse splenocytes at 28 days after immunization with the recombinant proteins was analyzed by ELISpot. The number of antigen-specific IgG-secreting B cells was significantly increased in mice immunized with r0628 and rTbpA compared with the non-immunized group; conversely,

that in mice immunized with rBfr was not increased (Fig. 4A). IFN-γ- and IL-4-secreting T cells were analyzed by ELISpot. The numbers of antigen-specific IgG-secreting B cells and IFN-γ-secreting T cells were significantly increased in the immunized groups with recombinant proteins than in the negative control groups ($p < 0.05$) (Fig. 4B). However, the number of IL-4-secreting T cells was significantly increased only in the immunized group with rTbpA compared with the non-immunized group (Fig. 4C).

Discussion

Brucella abortus is an infectious disease of domestic cattle and wild animals with serious zoonotic disease. In this study, we investigated to identify new antigen candidates for diagnosis of brucellosis through the immunogenicities of three recombinant proteins (r0628, rBfr, and rTbpA) of *B. abortus* instead of LPS antigen. The genes coding the proteins were cloned and recombinant proteins expressed using the pCold TF expression system. The recombinant proteins were identified by SDS-PAGE and western blot assay at the expected size after purification using an anti-histidine column.

TNF-α and IL-6 are the major inflammatory cytokines to evaluate an immune response after stimulation of antigens. Numerous previous studies have shown the production of TNF-α and IL-6 after the stimulation and/or immunization with various antigens of *Brucella* spp. in vitro. In this study, r0628, rBfr, and rTbpA could elicit production of TNF-α and IL-6 from the RAW 264.7 cells after 4 and 8 h stimulation with the recombinant proteins. These results were similar to the previous studies [18, 31, 34]. In addition, the production of NO was increased at 24 h after stimulation with the three recombinant proteins. Wang *et al*. [32] reported that live *B. abortus* induces low production of NO by RAW 264.7 cells. The production of IFN-γ and IL-2 was high at 24 h after stimulation with r0628 and rTbpA in splenocytes from naive mouse; conversely, the rBfr-stimulated group showed a very low production level of these cytokines. These results imply that r0628 and rTbpA triggered Th1 cell-mediated immune response by secreting IFN-γ and IL-2. The observation made from this study, where IFN-γ production was higher than IL-2, correlates to the study reported by Okamura *et al*. [26] in which the level of IFN-γ was increased due to Th1 cell activation by cytokines such as IL-12 [26]. Moreover, the production of IL-4 was not increased after stimulation with the recombinant proteins in this study. The study done by Fernández-Lago *et al*. [11] did not show a production of IL-4 in spleens after being infected with *B. abortus* 2308, which was the same case in this study. These results verified that high IFN-γ production in splenocytes could be induced by LPS-free antigens [4, 17, 31]. IFN-γ is one of the major cytokines evaluated in the immune response in vitro after stimulation or immunization with the antigens.

Mice immunized mice with the three recombinant proteins produced antigen-specific IgM and IgG sufficiently (Fig. S1). The production of antigen-specific IgM was induced at an early stage compared with antigen-specific IgG production. In particular, rTbpA showed strong immunogenicity after stimulation and/or immunization in both in vitro and in vivo tests. This antigen is one of the substrate-binding proteins. It is classified by the domain, and is associated with the membrane protein complex for transport into the cells [3]. In addition, IgG1 and IgG2a production was also increased in a time-dependent manner (Fig. S2). It indicated that the immunogenicity of proteins showed humoral (Th2) and cellular (Th1) immune responses in vivo. The production of antigen-specific IgG-secreting B cells and IFN-γ- or IL-4-secreting T cells in the spleen was determined from mice immunized with r0628, rBfr, and rTbpA. Two antigens, r0628 and rTbpA, significantly induced the production of B cells through the detection of antigen-specific IgG by ELISA. The rTbpA-immunized mice group showed a higher production of IL-4-secreting T cells, Th2-related, than the other groups. These results show that r0628 could induce memory T cells of the Th1 phenotype predominantly, and is in agreement with that of *B. abortus* infection in ASCs, which consist of macrophage and dendritic cells, and released cytokines such as TNF-α, IL-1β, and IL-6. Additionally, *Brucella* infection is affected by Th1 and/or Th2 cytokine balances in a mouse strain [16]. As the immunological outcome of r0628 or rTbpA had not been studied before, the results of this study would contribute to the understanding of the notable immunogenicity of r0628 and rTbpA as candidates of LPS-free antigens. Although there is already functional information known of cytokines in vitro, it is very rare for cytokines to act alone in vivo [19]. Therefore, the factors arising from the differences in the two environments contribute to such discrepancies between the in vitro and in vivo results.

In conclusion, our study provides an evaluation of the immunogenicities of three recombinant proteins (r0628, rBfr, and rTbpA) of *B. abortus* in terms of various immune responses. The antigens induced the production of NO, TNF-α, and IL-6 in RAW 264.7 cells after stimulation. Among them, r0628 and rTbpA showed strong IFN-γ

production in splenocytes. Therefore, this study suggests that those recombinant proteins could be a candidate for diagnostic markers. Further investigation on the role of r0628 and rTbpA will help in understanding the pathogenesis of *Brucella*.

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References

1. Al Dahouk S, Nockler K, Scholz HC, Tomaso H, Bogumil R, Neubauer H. 2006. Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infection. *J. Immunol. Methods* **309:** 34-47.
2. Baldi PC, Giambartolomei GH, Goldbaum FA, Abdon LF, Velikovsky CA, Kittelberger R, Fossati CA. 1996. Humoral immune response against lipopolysaccharide and cytoplasmic proteins of *Brucella abortus* in cattle vaccinated with *B. abortus* S19 or experimentally infected with *Yersinia enterocolitica* serotype O:9. *Clin. Diagn. Lab. Immunol.* **3:** 472-476.
3. Berntsson RP, Smits SH, Schmitt L, Slotboom DJ, Poolman B. 2010. A structural classification of substrate-binding proteins. *FEBS Lett.* **584:** 2606-2617.
4. Cha SB, Rayamajhi N, Kang ML, Lee WJ, Shin MK, Yoo HS. 2010. Comparative study of gamma interferon production in mice immunized with outer membrane proteins and whole bacteria of *Brucella abortus*. *Jpn. J. Infect. Dis.* **63:** 49-51.
5. Chaudhuri P, Prasad R, Kumar V, Gangaplara A. 2010. Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis. *Mol. Cell. Probes* **24:** 142-145.
6. Cloeckaert A, Jacques I, Bosseray N, Limet JN, Bowden R, Dubray G, Plommet M. 1991. Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*. *J. Med. Microbiol.* **34:** 175-180.
7. Connolly JP, Comerci D, Alefantis TG, Walz A, Quan M, Chafin R, *et al*. 2006. Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. *Proteomics* **6:** 3767-3780.
8. Denoel PA, Crawford RM, Zygmunt MS, Tibor A, Weynants VE, Godfroid F, *et al*. 1997. Survival of a bacterioferritin deletion mutant of *Brucella melitensis* 16M in human monocyte-derived macrophages. *Infect. Immun.* **65:** 4337-4340.
9. Detilleux PG, Deyoe BL, Cheville NF. 1990. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. *Vet. Pathol.* **27:** 317-328.
10. Detilleux PG, Deyoe BL, Cheville NF. 1990. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. *Infect. Immun.* **58:** 2320-2328.
11. Fernández-Lago L, Monte M, Chordi A. 1996. Endogenous gamma interferon and interleukin-10 in *Brucella abortus* 2308 infection in mice. *FEMS Immunol. Med. Microbiol.* **15:** 109-114
12. Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred host. *Int. J. Syst. Evol. Microbiol.* **57:** 2688-2693.
13. Franco MP, Mulder M, Gilman RH, Smith HL. 2007. Human brucellosis. *Lancet Infect. Dis.* **7:** 775-786.
14. Ghasemi A, Jeddi-Tehrani M, Mautner J, Salari MH, Zarnani AH. 2014. Immunization of mice with a novel recombinant molecular chaperon confers protection against *Brucella melitensis* infection. *Vaccine* **32:** 6659-6666.
15. Ghasemi A, Zarnani AH, Ghoodjani A, Rezani S, Salari MH, Jeddi-Tehrani M. 2014. Identification of a new immunogenic candidate conferring protection against *Brucella melitensis* infection in mice. *Mol. Immunol*. **62:** 142-149.
16. Golding B, Scott DE, Scharf OL, Huang LY, Zaitseva M, Lapham C, *et al*. 2001. Immunity and protection against *Brucella abortus*. *Microbes Infect.* **3:** 43-48.
17. González-Smith A, Vemulapalli R, Andrews E, Oñate A. 2006. Evaluation of *Brucella abortus* DNA vaccine by expression of Cu-Zn superoxide dismutase antigen fused to IL-2. *Immunobiology* **211:** 65-74.
18. Guillermo HG, Zwerdling A, Cassataro J, Bruno L, Fossati CA, Philipp MT. 2004. Lipoprotein, not lipopolysaccharide, are the key mediators of proinflammatory response elicited by heat-killed *Brucella abortus*. *J. Immunol.* **173:** 4635-4644.
19. Kindt TJ, Goldsby RA, Osborne BA. 2007. *Kuby Immunology*, pp. 306-307. 6th Ed. W.H. Freeman and Company, New York, NY.
20. Kittelberger R, Bundesen PG, Cloeckaert A, Greiser-Wilke I, Letesson JJ. 1998. Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9. *Vet. Microbiol.* **60:** 45-51.
21. Ko J, Splitter GA. 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. *Clin. Microbiol. Rev.* **16:** 65-78.
22. Ko KY, Kim JW, Her M, Kang SI, Jung SC, Cho DH, Kim JY. 2012. Immunogenic proteins of *Brucella abortus* to minimize cross reactions in brucellosis diagnosis. *Vet. Microbiol.* **156:** 374-380.
23. Lee JJ, Simborio HL, Reyes AW, Kim DG, Hop HT, Min W, *et al*. 2015. Immunoproteomic identification of immunodominant antigens independent of the time of infection in *Brucella abortus* 2308-challenged cattle. *Vet. Res.* **46:** 17.
24. Lim JJ, Kim DH, Lee JJ, Kim DG, Min W, Lee HJ, *et al*. 2012. Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of bovine brucellosis in Korea. *J. Vet. Med. Sci.* **74:** 687-691.
25. Nielsen K, Smith P, Yu WL, Halbert G. 2007. *Salmonella enterica*

serotype Urbana interference with brucellosis serology. *J. Immunoassay Immunochem.* **28:** 289-296.
26. Okamura H, Kashiwamura S, Tsutsu H, Yoshimoto T, Nakanish K. 1998. Regulation of interferon-gamma production by IL-12 and IL-18. *Curr. Opin. Immunol*. **10:** 259-264
27. Oñate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H. 1999. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. *Infect. Immun.* **67:** 986-988.
28. Ruiz-Mesa JD, Sanchez-Gonzalez J, Reguera JM, Maritin L, Lopez-Palmero S, Colmenero JD. 2005. Rose Bengal test: diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic area. *Clin. Microbiol. Infect.* **11:** 211-215.
29. Scholz HC, Nöckler K, Göllner C, Bahn P, Vergnaud G, Tomaso H, *et al*. 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int. J. Syst. Evol. Microbiol.* **60:** 801-808.
30. Simborio HL, Lee JJ, Bernardo Reyes AW, Hop HT, Arayan LT, Min W, *et al*. 2015. Evaluation of the combined use of the recombinant *Brucella abortus* Omp10, Omp19 and Omp28 proteins for the clinical diagnosis of bovine brucellosis. *Microb. Pathog.* **83-84:** 41-46.
31. Sung KY, Jung M, Shin MK, Park HE, Lee JJ, Kim S, Yoo HS. 2014. Induction of immune responses by two recombinant proteins of *Brucella abortus*, outer membrane proteins 2b porin and Cu/Zn superoxide dismutase, in mouse model. *J. Microbiol. Biotechnol.* **24:** 854-861.
32. Wang M, Qureshi N, Soeurt N, Splitter G. 2001. High levels of nitric oxide production decrease early but increase rate survival of *Brucella abortus* in macrophage. *Microb. Pathog.* **31:** 221-230.
33. Whatmore AM, Davison N, Cloeckaert A, Al Dahouk S, Zygmunt MS, Brew SD, *et al*. 2014. *Brucella papionis* sp. nov., isolated from baboons (*Papio* spp.). *Int. J. Syst. Evol. Microbiol.* **64:** 4120-4128.
34. Zhan Y, Kelso A, Cheers C. 1993. Cytokine production in the murine response to *Brucella* infection or immunization with antigenic extracts. *Immunology* **80:** 458-464.