수의학석사학위논문

# Analysis of *in vitro* and *in vivo* immunogenicities of *Brucella abortus* recombinant proteins, Omp2b and SodC

## *Brucella abortus*의 Omp2b와 SodC 재조합 단백질의 *in vitro*와 *in vivo* 면역원성의 분석

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Zoonotic Animal Disease
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**A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Master in Interdisplinary Program in Zoonotic Animal Disease**

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## Abstract

# Analysis of *in vitro* and *in vivo* immunogenicities of *Brucella abortus* recombinant proteins, Omp2b and SodC

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*Brucella abortus* is facultative intracellular bacterium and causative agent of bovine brucellosis. This zoonotic disease causes abortion and infertility in cattle and undulant fever, endocarditis, arthritis and osteomyelitis in humans. Diagnosis of *B. abortus* is mainly based on serological methods using antibody against LPS. However, several problems have been raised in LPS based diagnostic methods. Therefore, developing new protein antigen-based diagnostic reagent is required.

Based on the current knowledge, two proteins of *B. abortus*, Cu/Zn superoxide dismutase (SodC) and outer membrane proteins 2b porin (Omp2b) which were reported as immune-reactive proteins antigen candidates were selected to identify immunogenicities *in vitro* and *in vivo*. The recombinant proteins, rOmp2b and rSodC were expressed and purified in pMAL vector system as fusion forms with maltose-binding protein. The proteins were identified by SDS-PAGE and Western blot with sera of mice infected with *B. abortus*.

Murine macrophage RAW 264.7 cells were stimulated with the recombinant proteins to investigate production of cytokines and nitric oxide (NO). The two groups of murine macrophage RAW 264.7 cells produced TNF-α, IL-6 and NO after stimulation. BALB/c mice splenocytes were stimulated with the recombinant proteins to investigate production of cytokines. The two groups of splenocytes produced more IFN-γ than IL-4 after stimulation.

BALB/c mice were immunized with the recombinant proteins to investigate productions of antigen specific immunoglobulines in sera and number of cytokines producing cells in splecnocytes. The two groups of BALB/c mice produced antigen specific IgG and IgM in sera after immunization. However, the rOmp2b immunization group produced the immunoglobulines levels higher and earlier than the rSodC immunization

group. The two groups of BALB/c mice splenocytes increased the number of antigen specific IgG, IFN-γ producing cells. However, IL-4 producing cells in splenocytes only increased in the rOmp2b immunization group.

These results suggest that rOmp2b and rSodC can induce innate immune responses, Th-1 cell mediated immune responses and antigen specific antibodies production in mice. Also, rOmp2b have more immunogenicity than rSodC. Therefore, rOmp2b and rSodC considered as potential protein antigens in the serologic diagnosis of bovine brucellosis.

**Keywords**: *Brucella abortus*, Omp2b, SodC, immunogenicity, cytokine, antibody, ELISA

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# List of Abbreviations

| | |
|---|---|
| AP | Alkaline phosphatase |
| ASC | Antibody secreting cell |
| CSC | Cytokine secreting cell |
| CT | Complement test |
| DNA | DeoxyriboNucleoic Acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISPOT | Enzyme-linked immunospot |
| FBS | Fetal bovine serum |
| HKBA | Heat-killed *Brucella abortus* |
| IPTG | Isopropyl-β-D-1-thiogalactopyranoside |
| KTCC | Korea Type Culture Collection |
| IACUC | Institutional of Animal Care and Use Committee |
| LPS | Lipopolysaccharide |
| LB | Luria-Bertani |
| MBP | Maltose binding protein |
| NO | Nitric oxide |
| NED | N-(1-naphthyl) ethylenediamine dihydrochloride |
| OD | Optical density |
| Omp2b | Outer membrane protein group 2 |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| RBT | Rose Bengal test |
| RPMI1640 | Roswell Park Memorial Institute 1640 |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SodC | Cu/Zn superoxide dismutase |
| SPSS | Statistical Package for Social Science |
| STAT | Standard tube agglutination test |
| TMB | Tetramethyl-benzidine |
| 2- DE | Two dimensional electrophoresis |

# Introduction

Brucellosis is a major zoonotic disease with public health importance worldwide and causes huge economic loss on livestock industry. The causative agent of brucellosis is a genus *Brucella* which is a group of facultative intracellular bacteria. The bacteria are small, Gram negative coccobacilli that lack capsules, endospores or native plasmids (Young, 2001). The genus consists of ten species according to 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* (rats), *B. microti* (rats), cetacean (*Brucella ceti*) and seals (*Brucella pinnipedialis*) (Godfroid et al., 2010). Zoonotic potential of *Brucella* species is variable: *B. melitensis*, *B. abortus* and *B. suis* are considered most pathogenic species for humans. *B. canis*, *B. ceti* and *B. pinnipdialis* also cause human brucellosis but comparatively low zoonotic potential (Xavier et al., 2010).

Infection of *B. abortus* shows different clinical signs. This zoonotic pathogen causes undulant fever, endocarditis, arthritis and osteomyelitis in humans and abortion and infertility in cattle (WHO, 2006). *B. abortus* organisms are present in the reproductive tissue, fetal fluids and also concentrate in the udders of animals that produce milk (WOAH, 2009). Therefore, predominant route of exposure for *B. abortus* between animals is

through ingestion or inhalation of organisms that are present in the products of parturition. Human brucellosis is usually transmitted from infected animals by direct contacting the bacteria or consumption of unpasteurized dairy products from infected animals (Hadush et al., 2013).

Best approaches to prevent and control both human and bovine brucellosis are eliminating infected animals and preventing the spread of the disease. Despite all the tools available to eliminate the disease, an effective surveillance system is the critical first step (Ragan, 2002). Thus, diagnosis of *B. abortus* is the most importance step to achieve the goal. The lipopolysaccharide (LPS) has been considered as the most important antigen during immune response in brucellosis and can elicit long lasting serological response in both vaccinated and infected animal (Baldi et al., 1996). Diagnosis of *B. abortus* in ruminant is mainly based on serological confirmation with LPS-based antigens. Most widely used standard serological tests are the standard tube agglutination test (STAT), the Rose Bengal plate agglutination test (RBPT), and the complement fixation test (CFT).

Although LPS elicits a strong humoral immune response, there are several problems using LPS-based diagnostic methods. *Brucella* O-polysaccharide of LPS is similar to that of various Gram negative bacteria such as *Yersinia enterocolitica* O:9 (Kittelberger et al., 1998), *Escherichia coli* O:166/O157

(Nielsen et al., 2004), *Salmonella urbana* (Nielsen et al., 2007), *Francisella tularensis*, (Al Dahouk et al., 2005) and *Vibrio cholera* (Carlos et al., 2006). Accordingly, specificity of LPS-based diagnostic methods in brucellosis is low due to the cross-reactivity. Besides, it is hard to differentiate infected and/or vaccinated animals (Ruiz-Mesa et al., 2005). For these reasons, developing LPS-free protein-based diagnostic reagents is important. Outer membrane proteins (Omps) of *Brucella* spp. have been received the attention as major immunoreactive components in the bacterial cells to develop new diagnostic method or vaccine (Cloeckaert et al., 1991).

Although role and size of each Omps are various, Omp2b as a bacterial component, porin has important roles in the bacterial living (Vizcaino & Cloekaert, 2012). Cu/Zn superoxide dismutase (SodC), cellular protein with antioxidant activities is also essential for *brucella* intracellular survival (Gee et al., 2005). Immunoproteomics is the approach to identify specific immunogenic proteins in high resolution in the wide range of proteins expressed by *brucella*. The Omp2b and SodC have been identified as immune-reactive proteins in proteomic analysis of *B. abortus* (Al Dahouk et al., 2006; Connoly et al., 2006; Ko et al., 2012). Based on the knowledge, the two proteins were selected to identify immunogenicity *in vitro* and *in vivo* in order to evaluate these proteins as LPS-free protein sero-diagnostic reagent candidates for bovine brucellosis.

# Literature Review

Brucellosis is a zoonotic infectious disease of domestic animals, wild animals and humans. It was first identified by David Bruce in the 1860s in Malta, which became known as Malta fever (Araj, 2010). Ten *Brucella* species are currently identified (Table 1). The causative organisms, *Brucella* species are small, Gram-negative, aerobic, facultative intracellular, coccobacilli bacteria and they invade and survive for long periods of time and multiply within host cell (Poester et al., 2013). Unlike other pathogenic bacteria, *Brucella* do not have well-known bacterial virulence factors such as exotoxins, cytolysins, capsules, fimbriae and plasmids (Delvecchio et al., 2002; Paulsen et al., 2002).

Infection of this bacterium to pregnant animal causes placentitis and abortion due to the trophism of the bacteria to trophoblasts, fetal lung, macrophages and reproductive organ (Adams, 2002). Therefore, the infection causes devastating economic effects on livestock production. The contacts with infected cattle as well as the consumption of unpasteurized milk from the animals are important routes for the transmission of *B. abortus* to humans (Godfroid et al., 2010).

Table 1. Zoonotic potential and host preference of *Brucella* species

| **Species** | **Zoonotic potential** | **Prefered host** |
|---|---|---|
| *Brucella melitensis* | High | Sheep, goat |
| *Brucella abortus* | Moderate | Cattle |
| *Brucella suis* | Moderate | Pig |
| *Brucella canis* | Mild | Dog |
| *Brucella ovis* | Absent | Sheep |
| *Brucella neotomae* | Absent | Desert wood rat (Neotomae lepida) |
| *Brucella ceti* | Mild | Cetaceans |
| *Brucella pinnipedialis* | Mild | Seals |
| *Brucella microti* | Absent | Common voles (microtus arvalis) |
| *Brucella inopinata* | Moderate | human |

Adapted from Xavier *et al*., 2010.

## Immunity of *Brucella* infection

The host immune response is functionally divided into innate and adaptive immunity. The innate immune system is the first line of defense against invading pathogens. Its elements include physical barriers (skin and internal epithelial layers), humoral components (various chemokines, complement system, and opsonins) and cellular components, such as phagocytes (neutrophils, monocytes, macrophages, dendritic cells) and innate lymphocyte subsets (natural killer cells and γδ T cells).

The adaptive immunity can be divided into cell-mediated immunity and humoral immunity. T lymphocytes play a major role in cell-mediated immunity by production of cytokines and cytotoxic effects. The major cells in humoral immunity are antibody-producing B lymphocytes (Parkin & Cohen, 2001).

Because *Brucella* spp. have ability to survive and replicate inside host cell especially macrophage, host protection against *Brucella* spp. depends on activated APCs in innate immunity and activated T helper cells and cytotoxic T cells in adaptive immunity to remove the organisms and infected cells (Baldwin & Goenka, 2006; Skendros et al, 2011).

# 1. Innate immunity

The innate immunity is rapid, non specific and non memory immune response against invading pathogens. It consists of physical barriers at the surface of the body, humoral components, such as complement proteins, and cellular components including macrophages, dendritic cells (DCs), granulocytes (basophils, eosinophils, and neutrophils), and natural killer cells (Dranoff, 2004).

## 1.1. Physical barriers

The first line of physical barrier is epithelial cells located in mucosal surface of intestine, genitourinary and respiratory tract of host. Intestinal epithelial cell not only block invading enteric pathogen but also trigger immune response like professional immune cells. The epithelial cells express receptors of innate immune system and can recognize microbial pathogen then produce proinflammatory mediators (Abreu, 2010). *Brucella* induces only a weak proinflammatory response in intestinal epithelial cells but produce a significant chemokine (C-C motif) ligand 20 (CCL20) (Gorvel et al., 2009).

Gastric juice of intestinal cavity can give extreme environmental such as low pH and digestive enzymes to enteric *Brucella* (Delpino et al., 2007).

Microfold cells (M cells) are found in the follicle associated epithelium of Peyer's patch and BALT (Bronchus-associated lymphoid tissue). They transport invading *Brucella* from the lumen to immune cells under the epithelial cells in order to stimulate innate immune cells by phagocytosis. (Paixão et al., 2009).

## 1.2. Cellular components

Cellular components including macrophages, DCs, neutrophils and innate T cells have major roles in innate immunity. They recognize invading *Brucella* and reduce the organism or infected cell by phagocytosis or cytotoxic activity. In addition, they can induce adaptive immunity through presenting the pathogens epitopes to adaptive immune cells and producing cytokines.

### 1.2.1. Antigen presenting cells (APCs)

Macrophage and DC are major APCs in innate immunity against *Brucella* infection. Both cell types have various inducible mechanisms to eliminate bacteria (Table 2). APCs are the earliest cells that react invading microbes and induction of adaptive immune response by presentation of epitope of antigen to T helper (Th) cells. The interaction was achieved by pattern recognition receptors (PRRs) of APCs with pathogen associated molecular

patterns (PAMPs) of invading microbes. Major PAMPs of *Brucella* including bacterial lipopolysaccharide (LPS), DNA and lipoprotein are recognized by PRRs of APCs. The PRRs consists of Toll-like receptor 4 (TLR4), TLR9 and TLR2 (Giambartolomei et al., 2004; Pasquevich et al., 2010). Recently, TLR6 has been known as requirement to trigger innate immune response against *B. abortus* in vivo (de Almeida et al., 2013) (Figure 1).

Binding of these PRRs with their target PAMPs activate the principal transcription factor such as nuclear factor (NF)-κB, activator protein (AP)-1, interferon (IFN) regulatory factor (IRF) 3/7. Thus, APC produce several cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-12 and IL-6 as well as express costimulation molecules, cluster of differentiation (CD)80 and CD86, part of the innate and adaptive immune system (Macedo et al., 2008; Delpino et al., 2012) (Figure 1).

IL-12 plays a critical role in downstream events including activation of innate immune cells, T and B cells, which differentiate into antigen-specific effector cells (Metzger et al., 1996; Orange & Biron, 1996) (Figure 2). APCs can process phagocyted bacteria to form peptides that can be loaded into major histocompatibility complex (MHC) class I and II molecules on cell surface. They stimulate the T cell via MHC-peptide complexes that trigger T cell receptors (TCRs) and costimulatory molecules, B7.1/2, that

triggers CD28 on the T cells (Golding et al., 2001) (Figure 3).

In addition to antigen presenting function, macrophages can kill invading bacteria by phagocyte activity. Stimulating macrophage with microbial products, IFN-γ and TNF-α trigger many antimicrobial activities, including production of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (Jiang & Baldwin. 1993; Jiang et al., 1993). Both RNIs and ROIs play a role in controlling *Brucella* infection in the early stages of infection (Ko & Splitter, 2003). Important roles of the Th1 cytokine, IFN-γ in activating macrophages and in limiting *Brucella* infections were proven in both in vitro and in vivo (Trinchieri, 1997). Conversely, the Th2 cytokine such as IL-10 can suppress macrophage function and increase susceptibility to infection to *Brucella* (Fernandes & Baldwin, 1995: Saraiva & O'Garra, 2010).

Table 2. Basic mechanisms of action of antigen-presenting cells against *Brucella.*

| **Effect mechanism** | **Mode of action** |
|---|---|
| Phagocytosis and autophagy | Degradation by hydrolytic enzymes of phagolysosomes/autolysosomes |
| Antimicrobial cationic peptides (defensins) | Direct killing |
| Oxidative burst | Direct killing by ROS |
| Cytokine production | |
| TNF-α | Strong enhancement of bactericidal activity of phagocytes |
| IL-12 | Priming Th1 immune response, leading to production of IFN-γ |
| Chemokine secretion (MCP-1, RANTES, MIP1a/MIP1b) | Migration immune cells and maintenance of inflammation to limit infection |
| Antigen presenting | Priming of specific immune response, leading to IFN-γ production and cytotoxicity |

CTL: cytotoxic lymphocyte
MCP: monocyte chemoattracted protein
RANTES: regulated and normal T cell expressed and secreted
MIP: microphage inflammatory protein
ROS: reactive oxygen species

Adapted from Skendros & Boura, 2013.

### 1.2.2. Neutrophils

Neutrophils are rapidly recruiting to the infection site and can kill microbes by phagocytosis, extracellular release of granule contents, cytokine secretion, and the formation of neutrophil extracellular traps (Brinkmann et al., 2004). Opsonization is required for neutrophils to ingest *Brucella* (Young, 2001). Lipoproteins of *Brucella* including lapidated outer membrane proteins 19 (L-Omp 19) can activated human neutrophils functions such as oxidative burst, neutrophil migration and neutrophil survival (Zwerdling et al., 2009). In contrast to macrophage, *Brucella* cannot replicate within neutrophils although it seems to resist killing (Barquero-Calvo et al., 2007).

### 1.2.3. Innate lymphocytes

Innate lymphocytes including natural killer (NK) cells, natural killer T (NKT) cell and γδ T cell are at the interface between innate and adaptive immunity. In comparison with antigen specific T cell, they are a smaller proportion of blood cell population and recognize non- peptide antigen without MHC restrict. The major role of innate lymphocyte is to produces IFN-γ before the expansion of specific Th1 responses (Kubota, 2010; Nyirenda et al., 2010). The γδ T cells show protection activity in early stage of human, bovine brucellosis (Kilic et al 2009; Skyberg et al., 2011).

Activated γδ T cells inhibit the growth of *Brucella* in macrophage through

a combination of mechanisms including granule- and Fas ligand-mediated cytotoxicity, macrophage activation via IFN-γ production and secretion of the potent bactericidal factors, granulysin and cathelicidin (Oliaro et al., 2005; Dudal et al., 2006). NK cells activated by IL-12 and TNF-α released from infected macrophage then kill the *Brucella* infected cells through cytotoxic activity. Additionally, NK cells have regulatory role on antibody response to *Brucella* (Baldwin & Winter, 1994; Gao et al., 2011).

## 1.3. Humoral components

Complement is a systemic plasma protein with various functions such opsonization by binding to antibodies or bacterial surfaces, or direct killing of the pathogens by forming a membrane attack complex causing the lysis of those bacteria (Robertson et al., 1998; Tizard, 2004). Both classical pathway and lectin pathway are involved in complement deposition and complement mediated killing of *Brucella*. Complement activity was closely related with antibody level in serum at early stage of *Brucella* infection. After early stage, complement is activated by the binding of mannose-binding lectin to carbohydrates on *Brucella* surfaces rather than antibody level. However, at the later stage of *Brucella* infection, the increased concentration of immunoglobulin causes prozone effects with complement

and cannot kill extracellular *Brucella* (Hoffmann & Houle, 1995: Fernandez-Prada et al., 2001). Interaction between *Brucella* and complement is mediated by LPS, the major surface component of *Brucella*. Because *B. abortus* LPS protects the organism from complement attacks, smooth strains of *B, abortus* are more resistant than LPS-deficient rough strains to complement (Eigenschenk et al., 1999).

Lysosome plays accessory role in the bactericidal actions of antibody and complement or directly acts through bacteriolysis and opsonization. However, comparing antibody titers of *B. abortus* positive bovine serum and bacteriolytic power, lytic activity of lysosome slightly decreased in high antibody leveled serum. Therefore, lysosome is not essential protection factor in *Brucella* infection (Gwakisa & Minga, 1992).

## 2. Adaptive immunity

The adaptive immunity is activated after presentation of epitope of antigen by APCs, innate immunity. Its antigen specific effect and immunological memory can eliminate the pathogens effectively and quickly. It consists of T helper cells, cytotoxic T cells and antigen specific antibody producing B cell. T cell activates bactericidal function in macrophage by producing cytokines such as IFN- γ or cytotoxic effects on infected cells. B cell produces antigen

specific antibody that neutralize or opsonize the antigen (Golding et al., 2001).

## 2.1. Cell-mediated immunity

The major immune cells in cellular immunity are $CD4^+$, T helper cells (Th) and $CD8^+$, cytotoxic T cells (Tc). Following activation by APCs, naïve Th0 cell differentiated in Th1 and Th2 subset by stimulation with IL-12 or IL-4, respectively. Tc cell become cytotoxic lymphocyte. Th1 cells primarily produce IFN-γ and IL-2 to activate cell mediated immunity whereas Th2 cells mainly produce IL-4, IL-5 and IL-10 to activate humoral immunity (Figure 2).

Th cells help B cells to produce immunoglobulins. Th1 response can activate IgG2 and IgG3 isotype switching, whereas Th2 response can promote IgG1 and IgE switching (Germain, 1994). These activations of T cells can limit or kill the invading *Brucella* directly and indirectly (Zhan & Cheers, 1995; Fernandes et al., 1996).

Th1 predominantly produce IFN-γ activating bactericidal function of macrophage to limit *Brucella* infection and TNF-α maximize this activating function (Zhan et al., 1996; Trinchieri, 1997; Brandão et al., 2012). In addition, IFN-γ promotes the expression of antigen present and costimulatory molecules on APCs, potentiates the apoptotic death of

*Brucella* infected macrophages (Yingst & Hoover, 2003; Martirosyan et al., 2011).

IL-12 produced by Th cells and APCs induces Th1 response to activate macrophage whereas IL-10 produced by T and B cell suppresses IFN-γ activated macrophage function and increase susceptibility to infection (Fernandes et al., 1995; Zhan & Cheers, 1995; Ko & Splitter, 2003). *B. abortus* infection induces IL-10 production to modulate macrophage as negative feedback control of IL-12 secretion (Fernandes et al., 1995; Fernandez-Lago et al., 1996; Xavier et al., 2013).

$CD8^+$, activated cytotoxic T-lymphocytes (CTLs) can also produce IFN-γ and reduce *Brucella* and infected macrophages through Fas- or perforin-mediated cytotoxicity (Oliveira & Splitter, 1995; Durward et al., 2010). Although *B. abortus* can induce CTLs even in the absence of $CD4^+$ function, The CTL mediated cytotoxicity can be activated by IFN-γ from other T lymphocyte (Skendros et al., 2011).

The $CD4^+$ cell, especially Th1 is more important to control *Brucella* infection than $CD8^+$ (Giambartolomei et al., 2002; Rafiei et al., 2006). Interestingly, during chronic/relapsing brucellosis in murin model and human clinical case, Th1 response decreased and CTLs activity increased in compensatory effect (Galdiero et al., 1995; Skendros et al., 2007). Other recent data from murine chronic brucellosis model indicates that CTLs also

decreased with Th1 cytokines (Durward et al., 2012).

In addition to primary role of innate immunity, polymorphonuclear leukocytes (PMNs) including neutrophils may have roles in the adaptive immunity. The absence of PMNs in *B. abortus* infected Genista, neutropenic mice showed more active adaptive immune response that revealed the unexpected negative influence of PMNs to adaptive immune response (Silva, 2010; Mantovani et al., 2011; Barquero-Calvo et al., 2013).

## 2.2. Humoral immunity

B lymphocyte produces antigen specific antibody through direct stimulation with antigens and costimulation from T cell and cytokines. However, B cell can produce antigen specific antibody by direct stimulation with *B. abortus* without T cell costimulation (Goldings et al., 2001). The antibody can neutralize the antigens and acts as opsonins that facilitate the phagocytosis of bacteria. Additionally, the antibody can activate complement to bind the microbes and promotes antibody dependent cell mediated cytotoxicity (ADCC) by macrophages, neutrohphils and NK cells (Skendros & Bourn, 2013).

Sera from *B. abortus* infected cattle generally contain antibodies against LPS covering outer surface of the bacteria. Antibody isotype switch requires the collaboration between B cells and Th cells. Because the Th1 immune

response is predominant in *Brucella* infection, the dominant IgG isotype is IgG2. The antibody response to *B. abortus* in cattle consists of early production of IgM, almost immediately production of IgG2 and later small amount of IgG1 and IgA (Elzer et al., 1994; Goenka et al., 2012).

Although passive transfer of *B. abortus* specific antibody could protect mice from *B. abortus* infection (Michaux-Charachon et al., 1997), in bovine brucellosis, the humoral immunity only limits *Brucella* at initial stage of infection and has little protective effects on the intracellular course of infection (Baldwin & Goenka, 2006; Bellaire et al., 2005). Moreover, the high concentration of IgG disturbs extracellular bactericidal effects mediated by complement and enhances intracellular location of bacterial resulting extension of brucellosis (Hoffmann & Houle, 1995).

The B cells play immuno-regulatory role in brucellosis, producing IL-10 and transforming growth factor (TGF) β, which attenuate IFN-γ mediated Th1 responses. Also, *B. abortus* expresses the virulence factor proline racemase PrpA that induces secretion of IL-10 from B cell (Spera et al., 2006: Goenka et al, 2011).

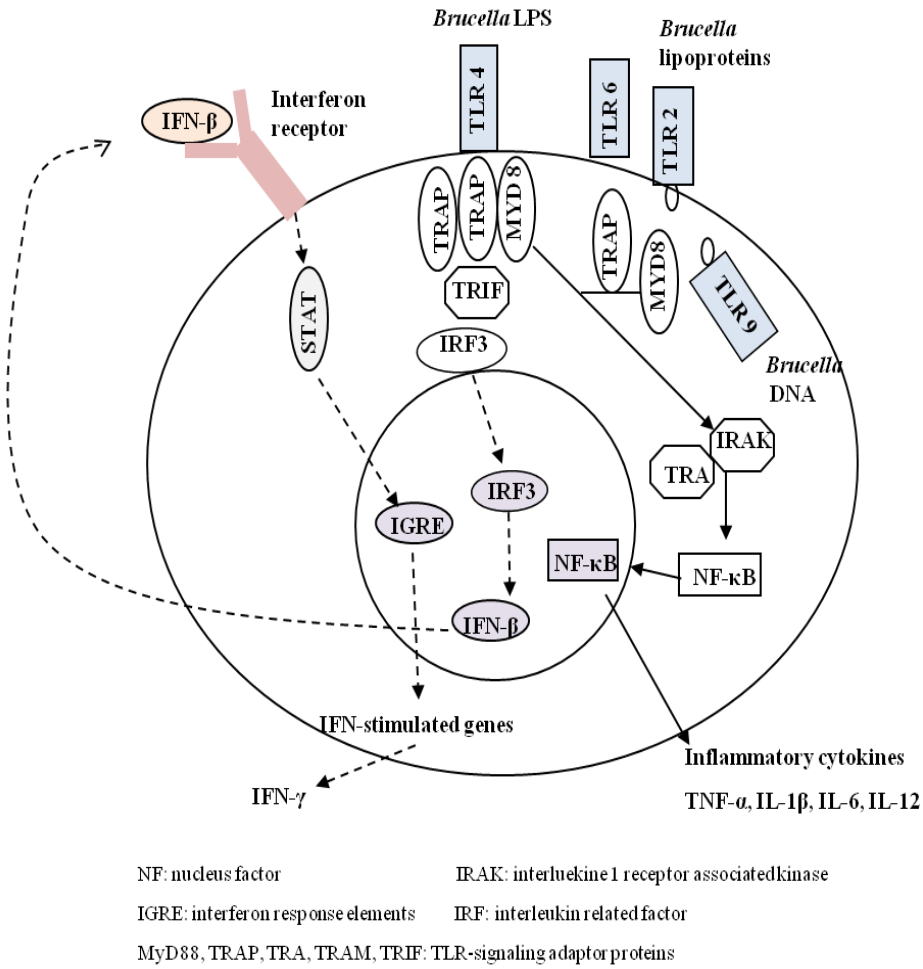


**Figure 1**. Overview of innate immunity signaling pathways in *Brucella* infection. Adapted from Olsen et al., 2010.

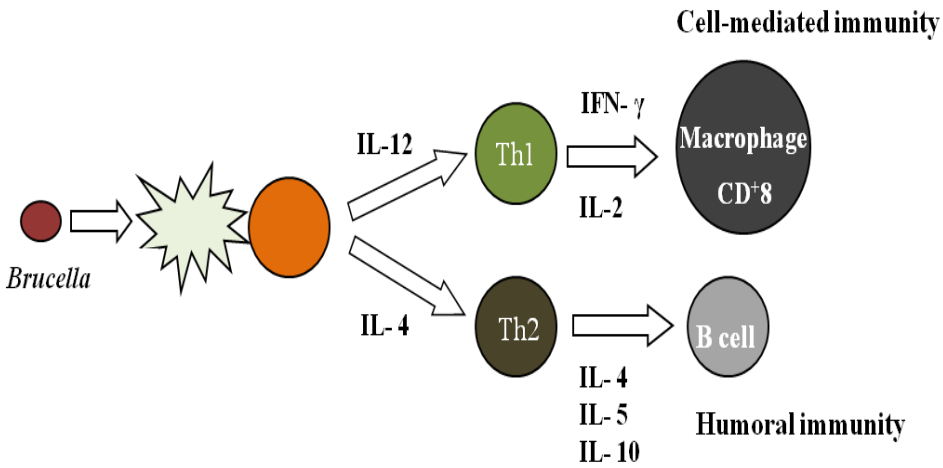


**Figure 2**. Differentiation of immune systems in *Brucella* infection. *Brucella* triggers APCs to release IL-12, which causes Th0 cells to differentiate into Th1 cells which secrete IFN-γ or IL-2. The Th1 cytokines enhance anti-*Brucella* mechanism of macrophage or induce the $CD^{+}8$ cytotoxicity. The Th2 response activates B cell for antibody production, facilitating the phagocytosis of *Brucella* through opsonization. The Th2 cytokines inhibit the action of Th1 cytokines. Adapted from Golding et al., 2001.

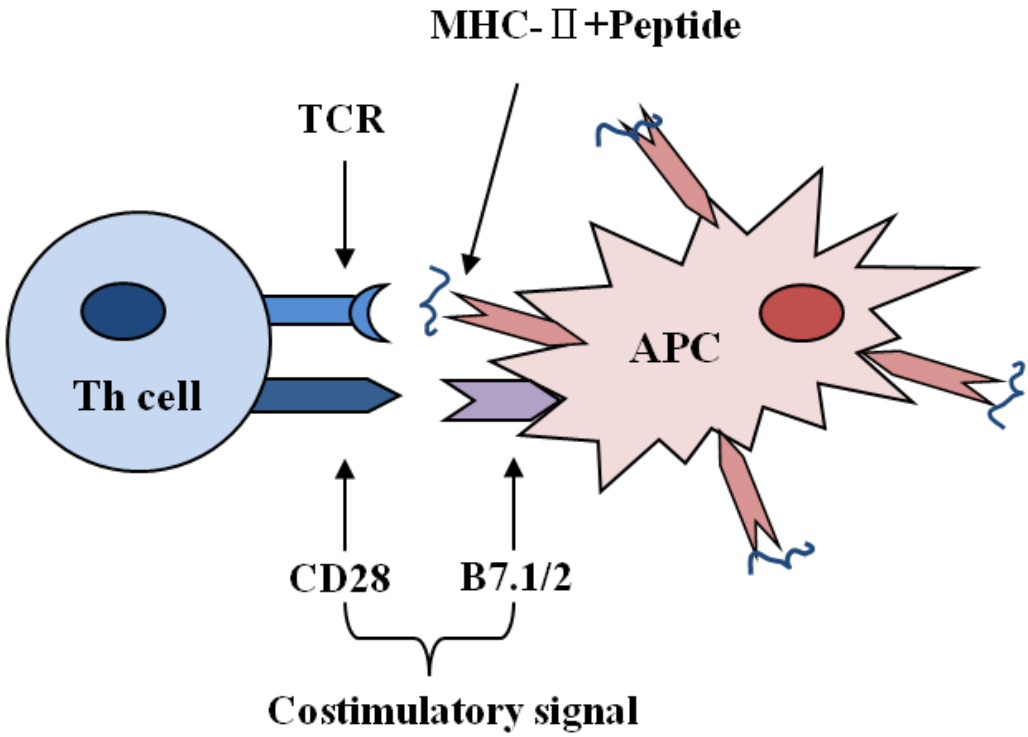


**Figure 3**. Diagram of the interactions between antigen presenting cell and Th cell. APCs deliver at least two signals to Th cells. One signal is via MHC–peptide on the APC that activates the TCR. The other is mediated by B7 molecules on the APCs which interact with CD28 on Th cells. Adapted from Golding et al., 2001.

# 3. Summary

*Brucellae* are intracellular bacteria that cause brucellosis. Physical barriers against *Brucella* infection consist of epithelial cells in respiratory, skin, intestinal tract and gastric juices. After the *Brucella* cross physical barrier, the bacteria activate innate immunity. APCs including macrophage and DC can detect invading the organisms by several TLRs signaling pathways and produce various inflammatory cytokines that activate both innate and adaptive immunity. Also, the signal activates macrophage to kill the *Brucella* by ROIs or RNIs. Together with the macrophage, neutrophils and innate T cell can reduce the infection by phagocytosis, cytotoxicity and production of inflammatory cytokines before adaptive immune response begin. In addition, complement system against *Brucella* can be activated with antibody dependent or independent ways for opsonization.

The APCs phagocyte the *Brucella* in order to present epitope to $CD4^+$ cell and activate $CD8^+$ cell for adaptive immunity. The activated APCs induce differentiation of $CD4^+$ cell to Th1, cellular immune response by IL-12 and Th2, humoral immune response by IL-4. The Th1 response leads to produce IFN-γ that enhances the clearance of infected cells and *Brucella* by macrophage and CTLs. Production of antibody against *Brucella* mainly consists of IgG2 which activated by Th1 immune response. Although the

antigen specific antibodies limit extracellular *Brucella* with complement activity in early stage, the humoral immune response is not protective in intracellular course of *Brucella* infection.

*Brucella* can manipulate cellular immunity or hide from the host immunity resulting in intracellular persistence by using various strategies. Therefore, understanding the interactions between *Brucella* and host immune system is first step to develop preventive and diagnostic methods against brucellosis.

# Materials and Methods

## 1. Recombinant proteins

Recombinant proteins expressed with pMAL vector were kindly provided by Professor Suk Kim, College of Veterinary Medicine, Gyeongsang National University. Briefly, total genomic DNA were prepared from *Brucella abortus* 544 culture using G-spin$^{tm}$ Genomic DNA Extraction kit for bacteria (Intron, Korea). Genes encoding Omp2 and SodC of *B. abortus* were amplified by PCR with primers pairs with appropriative restriction enzyme sites. The amplified DNA were digested with appropriated restriction enzymes and ligated into a pMAL vector (New England Biolab, USA). The recombinant plasmids were then transformed into the *E. coli* DH5α host cell for expression. The expression and purification of recombinant proteins were performed using Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, sonication, centrifugation, and Maltose resin (Bio-Rad, USA) column. The purified proteins were stored at 20 ℃.

## 2. SDS-PAGE and Western-blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed by previously described (Lim et al., 2012). Briefly, the purified recombinant proteins were diluted with Laemmli sample buffer (Bio-Rad, USA) and boiled for 10 min at 100 ℃. After electrophoresis, samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron, Korea). Proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane (Novex, USA) in transfer buffer with a constant current of 2 mA / $cm^2$ for 90 min using a iBlot© transfer device (Novex, USA). Membranes were blocked by incubation in 5% skim milk (Bio-world, Korea) for 1 h at room temperature (RT) and washed 3 times with washing buffer (TTSB) then incubated with *Brucella*-positive mouse sera (1:200 dilution) with 3 h at RT. The membranes were washed again 6 times per 5 min with the same buffer then incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000 dilution, Bethyl, USA) with 1 h at RT. After final washing, the proteins were visualized with AP conjugate substrate kit (Bio-Rad, USA).

## 3. Cytokines and nitric oxide (NO) measurement in a murine macrophage RAW 264. 7 cells

Murine macrophage RAW 264.7 cells were obtained from Korean Type Culture Collection (KTCC, Korea) and cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco, USA) media supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and penicillin (50 μg/ml, Sigma, USA) and streptomycin (50 μg/ml, Sigma, USA) at 37 ℃ under 5% $CO_2$. After incubating the cells for 18 h in the 12 well plates (Greiner Bio One, Germany) containing $1 \times 10^6$ cells/ml, they were stimulated with 10 μg/ml of rOmp2b and rSodC. Activity of LPS contaminated in the recombinant proteins was inhibited by incubation with polymixin B (100 μg/ml) for 30 min on ice before stimulation of macrophage and splenocytes. *E. coli* LPS (1 μg/ml, Sigma, USA) was used as positive control. While, purified Maltose-Binding protein (MBP, 10 μg/ml, New England Biolab, USA) and RPMI1640 media only were used as negative control. The culture supernatants were collected in 24 h after stimulation. Amounts of TNF-α and IL-6 were measured using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (eBioscience inc., USA). Production of nitric oxide (NO) was measured by measuring the nitrite accumulation with the Griess reaction as describe previously

(Sosroseno et al., 2002). Briefly, 100 μl aliquots of the culture supernatants were incubated with same volume of the solution containing 1% sulfanilamide (Sigma, USA) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED, Sigma, USA) in 2.5% phosphoric acid. Then, incubated 10 min at RT and the absorbance was measured at 540 nm. Nitrite concentrations in each well were calculated based on the standard curve generated with sodium nitrite (Sigma, USA).

## 4. Cytokines measurement in splenocytes of naïve mice

For the IFN-γ and IL-4 measurement assay, splenocytes ($1 \times 10^6$ cells/ml) isolated from healthy female, 5-weeks-old BALB/c mice (Orient-Bio, Korea) were stimulated with 10 μg/ml of rOmp2b, rSodC and MBP. After 24 h, amounts of IFN-γ and IL-4 in the culture supernatants were measured using the ELISA according to the manufacturer's instruction (eBioscience Inc., USA). Concanavalin A (1 μg/ml, Sigma, USA) was used as positive control whereas MBP (10 μg/ml) and the media were used as negative control. All stimuli were pretreated with polymixin B to avoid the activity of LPS contaminated with recombinant proteins. All care and handling of animals were performed with the approval of Seoul National University Institutional Animal Care and Use Committees (IACUC) and approval number is SNU-121129-4-1.

## 5. Measurement of IgG, IFN-γ and IL-4 secreting cells in mice splenocytes

Recombinant Omp2b and rSodC specific IgG memory B cell, IFN-γ and IL-4 secreting T-cells from spleen of immunized mice were measured by the Enzyme-Linked ImmunoSpot (ELISpot$^{PLUS}$) assay kit according to the manufacture's instruction (Mabtech AB, Sweden). For IgG memory B cells, 200 μl of rOmp2b and rSodC in PBS (50 μg/ml) were added to ELIspot plate after pretreatment with 70% ethanol and coated by incubation overnight at 4 $^{o}$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 plate and coated as described above. After the incubation, the plate were then extensively washed with PBS five times and blocked with RPMI1640 with 10% FBS for 30 min at RT. After remove the medium, 5 μg of rOmp2b and rSodC were added to the well and splenocytes isolated from mice at 28 days after first immunization added into the wells at $1 \times 10^5$ cells/well concentration. The plate was incubated at 37 $^{o}$C under 5% $CO_2$ for 24 h for IgG or 48 h for IFN-γ and IL-4. After remove the cells, 100 μl of biotinylated anti-IgG, IL-4 and IFN-γ antibodies in PBS containing 0.5% FBS (PBS-0.5% FBS) were added to each well. After incubation for 2 h at RT, the plates were washed and streptavidin-HRP in PBS-0.5% FBS

were added and incubated for 1 h at RT. Antibody secreting cells (ASC) and Cytokine secreting cells(CSC) were visualized upon addition of ready-to-use TMB substrate solution after washing the wells with PBS. The number of ASC and CSC were counted using Eli.Scan+ (A.EL.VIS, Germany). All samples were in triplicate.

## 6. Production of immunoglobulins in mice immunized with the recombinant proteins

The 6-week-old BALB/c female mice, approved by Seoul National University (SNU-121129-4-1), were immunized by the intra-peritoneal injection of 30 g of the purified recombinant proteins, rOmp2b and rSodC mixed with complete Freund's Adjuvant (CFA, Sigma, USA) on day 0 and with incomplete Freund's Adjuvant (IFA, Sigma, USA) on day 14. Sera for antibody response detection were obtained at 0, 3, 7, 14 and 28 days after the first immunization. Production of antibody was determined by ELISA using purified recombinant proteins as coating antigens. Briefly, 96 well microplates (Greiner bio one, Germany) were coated by incubation of the purified recombinant proteins, Omp2b and SodC ( 5 ng per well), in coating buffer (14.2 mM Na2CO3, 34.9 mM NaHCO3, 3.1 mM NaN3, and pH 9.6) overnight at 4 $^{o}$C. The plates were then blocked with 1% bovine serum

albumin (Sigma, USA) in a solution of PBS containing 0.1% Triton X-100 (PBST) for 2 h at 37 $^{o}$C. After washing with PBST, 1/200 diluted serum samples were added to the wells and incubated for 1 h at 37 ℃. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad, USA) and IgM (Jackson Immuno, USA) diluted 1: 2000 in PBS containing 1% BSA were used to detect IgG and IgM respectively. The color development was carried out using a 3,3',5,5'-Tetramethyl-benzidine (TMB) substrate (Sigma, USA). The absorbance was measured using an automatic microplate reader (Molecular Device Co., USA) at 420 nm. All samples were in triplicate.

## 7. Statistics

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

# Results

## 1. Analysis of recombinant proteins

Cloning of the Omp2b and SodC genes in the pMAL expression system led to the expression of a MBP fusion protein and then they were purified by maltose resin column. The SDS-PAGE profiles of purified rOmp2b and rSodC size approximately 78.5 kDa and 60 kDa but unexpected size bands revealed at 50 kDa and 40 kDa, respectively (Figure 4, A-1, B-1). To evaluate the immunoreactivity of the recombinant proteins, a Western blotting was performed. Both expected and unexpected size band of rOmp2b and rSodC showed sero-reactivity to *B. abortus* positive mouse serum but to the MBP did not (Figure 4, A-2, B-2).

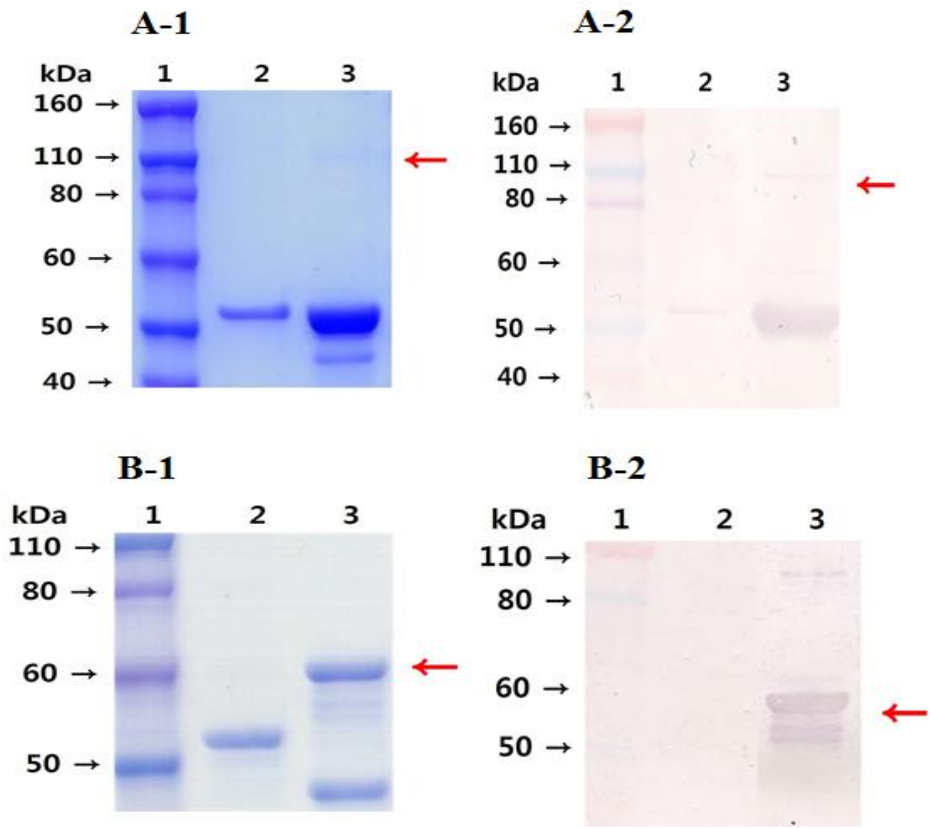


**Figure 4.** Analysis of purified recombinant Omp2b and SodC of *Brucella abortus.* SDS-PAGE (A-1) and Western blot (A-2) analysis of soluble protein fractions of pMAL-expressed Omp2b *Lane 1,* molecular weight markers, *Lane 2,* Purified MBP protein, *Lane 3,* Purified MBP-fusion protein. SDS-PAGE (B-1) and Western blot (B-2) analysis of soluble protein fractions of pMAL-expressed SodC *Lane 1,* molecular weight markers, *Lane 2,* Purified MBP protein, *Lane 3,* Purified MBP-fusion protein. The molecular weight of MBP-fusion proteins sizes were 78.5 kDa (Omp2b) and 60 kDa (SodC).

## 2. Production of TNF-α, IL-6 and NO in murine macrophage RAW 264.7 cells

Culture supernatants of murine macrophage RAW 264.7 cells were assayed for TNF-α, IL-6 and NO production at 24 h after stimulation with rOmp2b and rSodC by ELISA and Griess assay. Amount of TNF-α from the stimulating groups with two recombinants proteins were higher than those from the group stimulated with MBP ($P < 0.01$) (Figure 5A). Similarly, significant amount of IL-6 in the recombinant proteins exposure group was produced ($P < 0.01$) (Figure 5B). Production of NO in the experimental groups stimulated with the two recombinant proteins was also significantly higher than MBP control group ($P < 0.01$) (Figure 5C). Thus, the two recombinant proteins were proven the immune-stimulating activities in murine macrophage RAW 264.7 cells by production of TNF-α, IL-6 and NO.

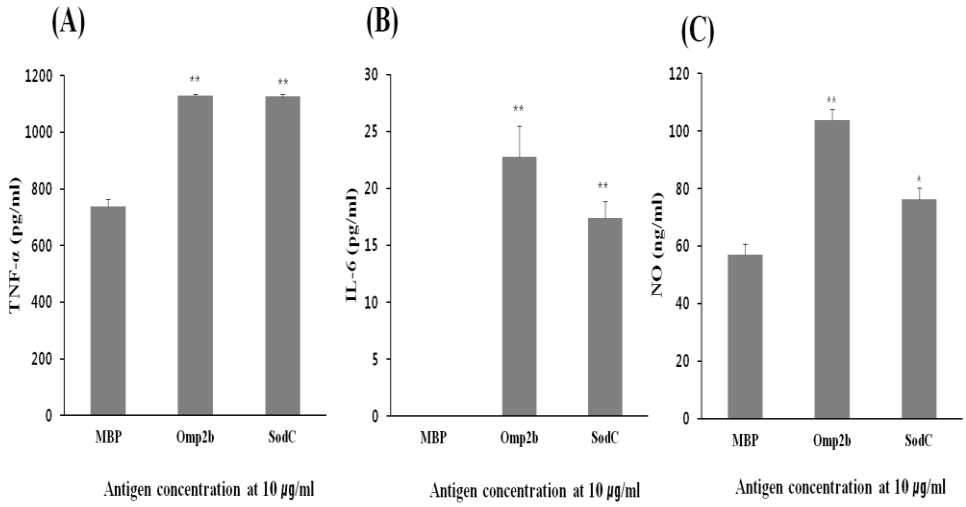


**Figure 5**. Production of TNF-α, IL-6, and NO from murine macrophage RAW 264.7 cells stimulated with purified rOmp2b and rSodC of *Brucella abortus* at 24h post stimulation (*, $P < 0.05$; **, $P < 0.01$). Maltose-binding protein (MBP) was used as vector control. The recombinant proteins were pre-treated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

## 3. Production of cytokines in naïve mice splenocytes

Amount of IFN-γ and IL-4 in naïve mice splenocytes culture supernatants were measured at 24 h after stimulation with the two recombinant proteins by ELISA. Production of IFN-γ and IL-4 from the mice splenocytes stimulated with rOmp2b and rSodC were higher than those from control group ($P < 0.01$) (Figure 6A). But production of IL-4 in MBP stimulated group was unable to detect ($P < 0.01$) (Figure 6B). The two proteins have significant effect to induce the production of IFN-γ and IL-4 compared with MBP alone.

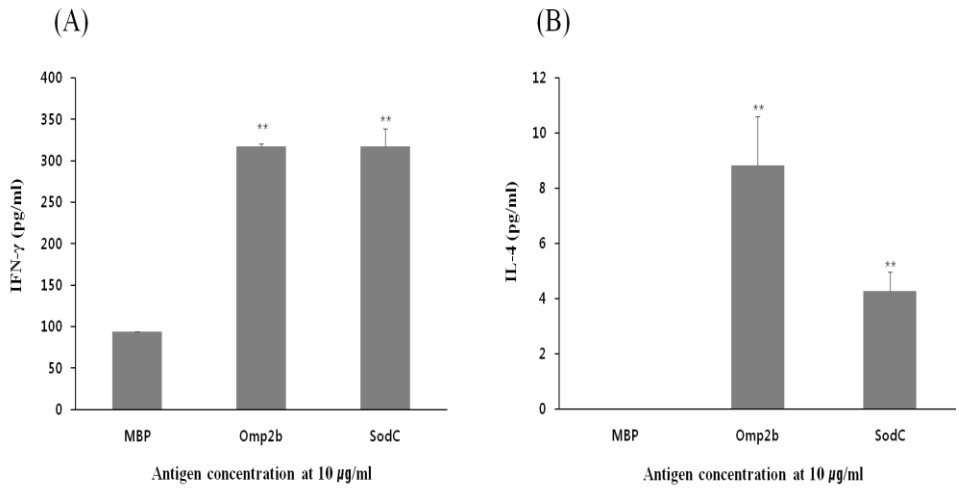


**Figure 6.** Production of IFN-γ and IL-4 from naïve mice splenocytes stimulated with purified rOmp2b and rSodC at 24h post stimulation (*, $P < 0.05$; **, $P < 0.01$). Maltose-binding protein (MBP) was used as vector control. The recombinant proteins were pre-treated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

## 4. Analysis of immune cells in splenocytes of mice immunized with rOmp2b and rSodC

Number of antigen specific IgG secreting B cells and IL-4 and IFN-γ secreting T cells from the mice splenocytes at 28 day after immunization were analyzed by ELISpot. The number of antigen-specific IgG secreting B cells and IFN-γ secreting T cells were significantly increased in immunization groups than negative control groups ($P < 0.05$) (Figure 7A and 7B). However, the number of IL-4 secreting T cells was significantly increased in only Omp2b immunization group compared with negative control group (Figure 7C).

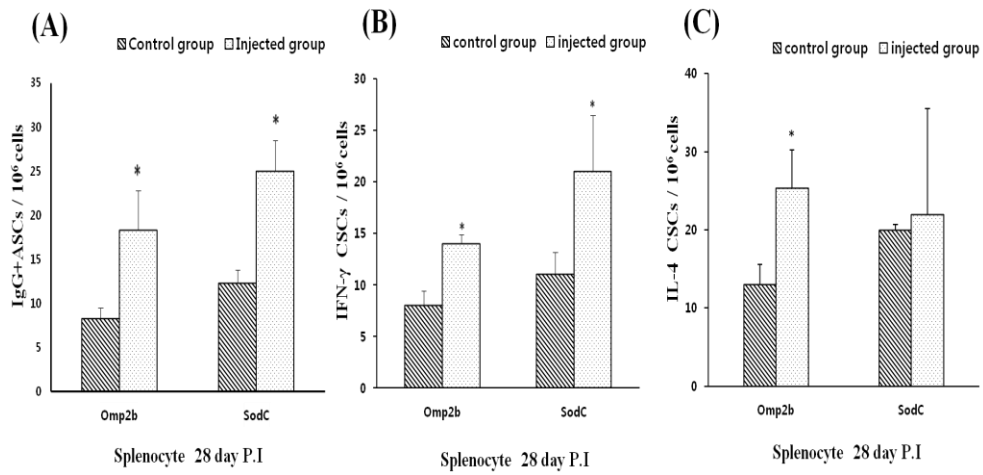


**Figure 7.** Number of antigen-specific immune cells from splenocytes of mice immunized with rOmp2b and rSodC of *Brucella abortus* at 28 days after immunization. Antigen specific IgG (A), IFN-γ (B) and IL-4 (C) secreting cells were measured by ELISpot after re-stimulation of splenocytes from immunized mice with the homologous antigens. The recombinant proteins were pre-treated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

## 5. Production of immunoglobulins in mice immunized with rOmp2b and rSodC

In the analysis of antibody production in mice by immunization with the two recombinant proteins, earlier and higher productions of IgG and IgM were observed in the groups immunized with Omp2b compared with other groups (Figure 8). Antibody production against SodC was slow in both IgG and IgM compared with Omp2b group. However, the final amount of IgG production in SodC group was similar with Omp2b group even though the response in SodC group was slow and later (Figure 8). Amount of IgM in SodC group was significantly slower and lower than those in Omp2b group (Figure 8).

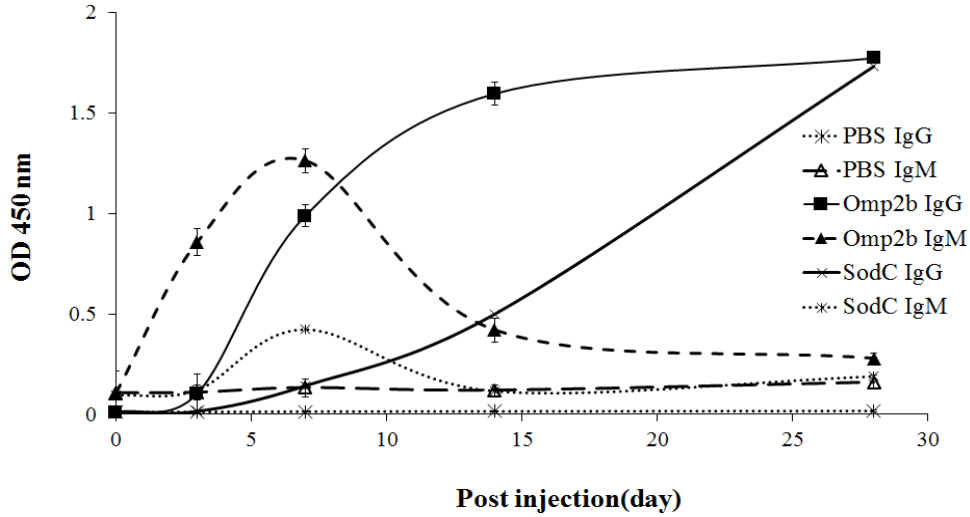


**Figure 8.** Humoral immune responses induced in mice by immunization with rOmp2b and rSodC of *Brucella abortus*. BALB/c mice (n=5) were inoculated intraperitoneally with the purified recombinant and adjuvant at two week interval. Serum samples were collected at different day points after the first immunization and IgG and IgM titers were measured by ELISA.

## Discussion

*Brucella abortus* is an infectious disease of domestic cattle and wild animals with serious zoonotic implication in humans. In this study, we investigated the immunogenicities of recombinant Omp2b and SodC of *B. abortus* to discover new LPS-free proteins antigen candidate for diagnosis of bovine brucellosis. The genes coding the proteins were cloned and expressed recombinant proteins using the pMAL expression system. The purified MBP fusion recombinant proteins were identified by SDS-PAGE and Western blot at expected size. However, unexpected proteins also identified at from 45 to 55 kDa size both rOmp2b and rSodC lanes. These were considered that the protein of interest might be in the wrong translational reading frame and MBP sized band would be produced by translational termination at the first in-frame stop codon. Or, MBP sized breakdown products were produced due to the instability of target proteins (Riggs. 2000).

In present study, rOmp2b and rSodC could elicit production of TNF-α and IL-6 from the murine macrophage RAW 264.7 cells after 24 h stimulated with recombinant proteins. These results are in agreement with the previous studies that *B. abortus* could induce in variety of cell types the release of pro-inflammatory cytokine such as IL-6 (Zhan et al,, 1993) and TNF-α

(Zhan & Cheers, 1995). In addition, heat-killed *brucella abortus* (HKBA) induced production of TNF-α and IL-6 from human monocyte cell-line (THP-1) and mouse peritoneal macrophage of both C3H/HeJ C3HeN mice (Giambartolomei et al., 2004). Weak production of NO in murine macrophage RAW 264.7 cells stimulated with rOmp2b and rSodC was in agreement with the previous paper that live *B. abortus* was poor at inducing NO production by murine macrophage RAW 264.7 cell (Wang et al., 2001).

After rOmp2b and rSodC *in vitro* stimulation, spleen cells from naïve mice produced high levels of IFN-γ and relatively low levels of IL-4. These results imply that rOmp2b and rSodC triggered Th1 cells to secrete IFN-γ, could induce Th1 cell-mediated immunity. In addition, rOmp2b could elicit Th2 cells to secrete IL-4 but lower than Th1 cell stimulation. These results were in accord with previous paper that *Brucella* Omps inoculated mice splenocytes showed high IFN-γ production (Cha et al., 2010) and rSodC induced sensitized $CD4^+$ IFN-γ producing cell, IFN-γ from splenocytes in BALB/c mice (Andrew et al., 2006; Fu et al., 2012).

Production of antigen-specific IgG secreting B cells and IFN-γ secreting T cells in splenocytes from 28 days after first immunized mice with rOmp2b and rSodC were higher than negative mice group. This results that rOmp2b and rSodC could induce memory T cells of the Th1 phenotype predominantly was in agreement with that immunity against *B. abortus*

infection is mainly cell associated (Golding et al., 2001) . Both rOmp2b and rSodC immunized mice produced antigen-specific IgM and IgG sufficiently. Interestingly, rOmp2b specific antibodies secreted more than rSodC group. The high number of antigen specific IgG secreting B cells of immunized splenocytes was in accord with humoral response of the mice. Although the rOmp2b has antigenicity in mouse model, Omp2b as major antibody targets in animal species has been controversial in previous papers (Cloekaert et al., 2002; Letteson et al., 1999). Therefore, further studies about antibody against Omp2b are needed. Production of rSodC specific antibodies in the immunized mice was in accord with previous papers (Onate et al., 1999; Fu et al., 2012). Until now, immunologic properties of Omp2b recombinant proteins have been poorly studied (Cloeckaert et al., 1996). Similarly, immunologic studies of SodC have mainly focused on develop vaccine candidate rather than diagnostic reagent (Andrew et al., 2006). Therefore, present study results contribute to understanding of notable immunogenicity of Omp2b and SodC for diagnosis purpose.

In conclusion, our recent study provides useful data for immunogenicities of Omp2b and SodC of *B. abortus* in terms of various immune responses. These recombinant proteins showed immunogenicities *in vitro* and *in vivo* especially in mouse model and it was similar with immunogenicity of *B. abortus* in host animals. Consequently, immunogenicities of rOmp2b and

rSodC considered these proteins as LPS-free antigen protein candidates, sero-diagnostic reagent for *B. abortus*. Further investigation about Omp2b and SodC needs to evaluate the potential for new diagnostic candidates for *B. abortus*.

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## 국문초록

# *Brucella abortus*의 Omp2b와 SodC 재조합 단백질의 *in vitro*와 *in vivo* 면역원성의 분석


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*Brucella abortus*는 그람 음성, 세포내 기생균이며 인수공통질병인 소 브루셀라증의 원인체이다. 소 브루셀라증의 혈청학적 진단은 주로 LPS에 대한 항체를 측정하는 방법을 사용하고 있다. LPS기반 진단법은 교차반응 등의 여러 문제점이 있으므로 이를 해결하기 위하여 새로운 단백질항원을 이용한 진단물질의 개발이 필요하다. 이번 연구에서는 소 브루셀라증의 혈청학적 진단을 위한 후보 단백질항원 개발을 위하여 기존 연구에서 면역반응성이 확인되고 *B. abortus*생존에 필수적인 역할을 하는 단백질인 Outer membrane proteins 2b porin (Omp2b)과 Cu/Zn superoxide dismutase (SodC)를 선택하여 실험동물 마우스에서 면역원성을 확인하였다.

재조합단백질 rOmp2b와 rSodC은 pMAL vector system에서 maltose-binding protein과 결합된 형태로 발현, 정제되어 SDS-PAGE와 *B. abortus*감염 마우스 혈청과의 Western blot을 통하여 확인되었다. 마우스를 이용한 *in vitro* 시험에서 재조합 단백질로 자극한 murine macrophage RAW 264.7세포에서 두 접종군 모두에서 TNF-α, IL-6와 NO의 유의적인 생성을 확인하였다. 재조합 단백질로 자극한 BALB/c mouse의 splenocytes에서 두 접종군 모두에서 IFN-γ가 IL-4보다 우세하게 생성되었다.

마우스를 이용한 *in vivo*시험에서 재조합 단백질을 접종한 BALB/c mouse혈청에서는 두 접종군 모두에서 항원 특이 IgG와 IgM항체를 생성 하였으나 rOmp2b접종군이 rSodC접종군 보다 조기에 더 많은 항체를 생성하였다. 재조합 단백질을 접종한 BALB/c mouse splenocytes는 두 단백질 접종군 모두에서 항원 특이 IgG및 IFN-γ생성 세포가 유의적으로 증가하였으나 IL-4생성세포는 rOmp2b 접종군에서 만 유의적으로 생성되었다.

이러한 결과들은 두 재조합 단백질 rOmp2b과 rSodC이 마우스에서 비특이 면역 반응, Th1 세포성 면역 반응 그리고 항체 생성 반응을 유발한다는 것을 나타내었으며 rOmp2b가 rSodC보다 좀 더

면역원성이 높음을 확인하였다. 따라서 rOmp2b와 rSodC는 소 브루셀라증에 대한 혈청 진단용 후보 단백질항원으로 유용할 것으로 여겨진다.

**핵심어 :** *Brucella abortus*, Omp2b, SodC, 면역원성, 싸이토카인, 항체, ELISA

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