Original Article
J Vet Sci 2018, 19(3), 416-425 · https://doi.org/10.4142/jvs.2018.19.3.416

Effect of immunization routes and protective efficacy of *Brucella* antigens delivered via *Salmonella* vector vaccine

Jonathan Lalsiamthara, Gayeon Won, John Hwa Lee*

College of Veterinary Medicine, Chonbuk National University, Iksan 54596, Korea

An anti-*Brucella* vaccine candidate comprised of purified *Brucella* lipopolysaccharide (LPS) and a cocktail of four *Salmonella* Typhimurium (ST)-*Brucella* vectors was reported previously. Each vector constitutively expressed highly conserved *Brucella* antigens (rB), *viz*., lumazine synthase (BLS), proline racemase subunit A, outer membrane protein-19 (Omp19), and Cu-Zn superoxide dismutase (SOD). The present study determined a relative level of protection conferred by each single strain. Upon virulent challenge, the challenge strain was recovered most abundantly in non-immunized control mice, with the ST-Omp19-, ST-BLS-, LPS-, and ST-SOD-immunized mice showing much less burden. Indirect enzyme-linked immunosorbent assay-based assay also confirmed the induction of antigen-specific immunoglobulin G for each antigen delivered. In a route-wise comparison of the combined vaccine candidate, intraperitoneal (IP), intramuscular (IM), and subcutaneous immunizations revealed an indication of highly efficient routes of protection. Splenocytes of mice immunized via IM and IP routes showed significant relative expression of IL-17 upon antigenic pulsing. Taken together, each of the *Brucella* antigens delivered by ST successfully induced an antigen-specific immune response, and it was also evident that an individual antigen strain can confer a considerable degree of protection. More effective protection was observed when the candidate was inoculated via IP and IM routes.

Keywords: *Salmonella* delivery, brucellosis, protective efficacy, vaccination

Introduction

Brucellosis is caused by the intracellular bacterial pathogen *Brucella*, comprising several species affecting man, companion animals, livestock, feral animals, and marine mammals [12,14]. In the absence of human anti-*Brucella* vaccine, animal vaccination is crucial, not only to protect animal health but also to stop zoonotic transmissions [3]. In an attempt to avoid non-ideal live *Brucella* organisms during vaccine preparation, we investigated the use of a highly attenuated rough *Salmonella* Typhimurium (ST) vector to deliver *Brucella* antigens into a critical niche comparable to natural *Brucella* infection. In our previous study, we reported an evaluation of a cocktail of engineered *Salmonella* vectors delivering heterologous *Brucella* antigens. Briefly, the selected antigen includes lumazine synthase (BLS), proline racemase subunit A (PrpA), lipoprotein outer membrane protein-19 (Omp19), and Cu-Zn superoxide dismutase (SOD) [8]. The BLS can induce a mixed Th1−Th2 pathway response, which accounts for humoral and cell-mediated immune responses [20], while the PrpA has a potent stimulatory effect on splenocytes and B-cells, and that enzyme is also associated with chronic infection of *Brucella* [18]. PrpA immunization can generate anti-PrpA antibodies in vaccinated animals, which, in turn, may attenuate the chronicity of *Brucella* infection. The Omp19 elicits adaptive interleukin (IL)-17 cytokine production, which is necessary for mucosal immunity, and it also shows protective efficacy while used as a subunit or a DNA vaccine platform [15], while the SOD is a protective antigen protein that is able to induce diverse immune responses [13]. This study investigated the immunostimulatory effects of *Brucella* lipopolysaccharide (LPS) combined with a live vaccine system. The complete *Salmonella*-based *Brucella* vaccine (ST-rB) formulation consists of a combination of ST strains in conjunction with purified *Brucella* LPS. It is important to determine whether any of the ST strains can be omitted from the combination without compromising the vaccine's protective efficacy. This information will allow formulation of a vaccine that is simpler and more economical to produce.

In this study, an engineered highly attenuated *Salmonella* vector was used for the purpose of vaccine antigen delivery. A

Received 24 Oct. 2017, Revised 4 Dec. 2017, Accepted 10 Jan. 2018
*Corresponding author: Tel: +82-63-850-0940; Fax: +82-63-850-0910; E-mail: johnhlee@jbnu.ac.kr
pISSN 1229-845X
eISSN 1976-555X

rough ST strain designated JOL1800 (Δ*lon*Δ*cpxR*Δ*asd*Δ*rfaL*) was electroporated with plasmids harboring the gene of interest. Stable maintenance of the plasmid was mediated by auxotrophic mutation of the *asd* gene. The *lon* and *cpxR* genes are involved in bacterial physiology and associated with virulency. The *waaL* gene (formerly *rfaL*) is involved in the biosynthesis of O-antigen, and thus, mutation of that gene rendered the bacterial strain a rough phenotype [4]. In this study, we evaluated the levels of protection conferred by those four individual *Brucella* antigen-*Salmonella* strains in a mouse model. In addition, we determined whether each antigen is delivered successfully to the host by confirming the presence of antigen-specific immunoglobulin G (IgG). To ascertain the most effective route of immunization, mice were administered with the complete ST-rB LPS vaccine via oral, intramuscular (IM), intraperitoneal (IP), and subcutaneous (SC) routes. Protective efficacy of each route was determined upon virulent challenge of mice groups with wild-type *Brucella abortus* 544.

Materials and Methods

Ethics and biosafety statement

All animal experimental procedures were approved (CBNU2015-00085) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with Animals). All mice used in the study were housed and maintained humanely and were provided water and antibiotic-free food *ad libitum*. The biosafety level-3 organism *B. abortus* was handled with appropriate safety precautions and under the supervision of Ministry of Health & Welfare, Korea.

Bacterial strains, plasmids, and primers

The bacterial strains, plasmids, and primers applied in this study are listed in Table 1. All *asd* gene-deleted *Salmonella* and *Escherichia coli* strains were incubated in Luria-Bertani (LB) medium supplemented with 50 μg/mL diaminopimelic acid. *B. abortus* strains RB51 and 544 were grown on BD *Brucella*

Table 1. Bacterial strains, plasmids, and polymerase chain reaction primers used in this study

Strain/plasmid/primer	Description	Reference
Strains		
JOL1800	JOL912 Δ*rfaL*; O-antigen deficient rough strain, bacterial delivery vector	Lab stock
JOL1818	JOL1800 harboring pJHL65 plasmid; *Salmonella* vector control	Lab stock
JOL1878	JOL1800 delivering heterologous *Brucella abortus* antigen, SOD	[8]
JOL1879	JOL1800 delivering heterologous *B. abortus* antigen, Omp19	[8]
JOL1880	JOL1800 delivering heterologous *B. abortus* antigen, BLS	[8]
JOL1881	JOL1800 delivering heterologous *B. abortus* antigen, PrpA	[8]
JOL1921	*Escherichia coli* BL21(DE3) pLysS expressing SOD via pET28a(+) system	This study
JOL1922	*E. coli* BL21(DE3) pLysS expressing Omp19 via pET28a(+) system	This study
JOL1923	*E. coli* BL21(DE3) pLysS expressing BLS via pET28a(+) system	[8]
JOL1924	*E. coli* BL21(DE3) pLysS expressing PrpA via pET28a(+) system	Lab stock
544	*B. abortus* strain 544, virulent challenge strain	Lab stock
RB51	*B. abortus* strain, rough attenuated live vaccine, reference strain	[16]
Plasmids		
pET28(+)	IPTG-inducible, T7 expression vector, C-terminal 6× histidine tag, Kan^R	Novagen
pJHL65	asd^+ plasmid, pBR ori, β-lactamase signal sequence-based periplasmic secretion plasmid, 6× His, high copy number plasmid	[7]
Primers		
BLS F	5′- GAATTCaaccaaagctgtccgaacaa; amplicon size, 486 bp	[8]
BLS R	5′- AAGCTTtcagacaagcgcggcgatgc	
PrpA EcoRI F	5′-GAATTCgcaagacattccttcttctgcg; amplicon size, 1011 bp	[8]
PrpA HindIII R	5′-AAGCTTtatgccatgctgaacccatgagca	
Omp19 EcoRI F	5′-GAATTCggaatttcaaaagcaagtctgctc; amplicon size, 543 bp	[8]
Omp19 HindIII R	5′-AAGCTTtcagcgcgacagcgtcacggc	
SOD EcoRI F	5′-CCGCGAATTCaagtccttatttattgcatcg; amplicon size, 519 bp	[6]
SOD HindIII R	5-CCGCAAGCTTtattcgatcacgccgcagg	

SOD, Cu-Zn superoxide dismutase; Omp19, outer membrane protein-19; BLS, *Brucella* antigens lumazine synthase; PrpA, proline racemase subunit A; F, forward; R, reverse.

medium in an aerobic or 5% CO_2 atmosphere. The constructed vaccine candidate is described elsewhere [8]. Briefly, the open reading frames (ORF) of PrpA, Omp19, and SOD were amplified from the strain 544 genome by using polymerase chain reaction (PCR), and then, inserted into the live vector pJHL65 via the incorporated *EcoR*I and *Hind*III restriction sites. The BLS gene cassette was synthesized *in vitro* due to incompatibility in the restriction enzyme. The pJHL-65 plasmid harbors the *asd* gene, *bla* secretion signal, 6× His tag sequence, and a Ptrc promoter. The plasmid was used to electrotransform the ST delivery strain JOL1800 (Table 1). *Brucella* LPS was extracted from smooth strain *B. abortus* 544 by using a phenol-based commercial LPS extraction kit (iNtRON Biotechnology, Korea).

Cloning and expression of recombinant *Brucella* protein in BL21 *E. coli* strain

The ORF of selected genes were amplified using the *pfu* high-fidelity enzyme and cloned in the pET28(+) plasmid. The plasmid was transferred to the BL21 *E. coli* expression system (Table 1). The expressing clones were induced with 1 mM IPTG at 0.6 optical density (OD) for 4 h. The culture was pelleted and subject to urea lysis and purification. The desired protein was purified by using a nickel-NTA column. The eluted purified protein was confirmed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity and the protein concentration was ascertained by using the Bradford protein assay (Biorad, USA). The recombinant protein was used as the antigen for indirect enzyme-linked immunosorbent assay (ELISA)-based detection of the delivered antigen-antibody response. Expression and purification of BLS and PrpA protein were described in our previous report [8]. After 4 h of induction with IPTG at 37°C, the conspicuous protein band corresponding to the desired protein was observed by performing Coomassie blue-stained SDS-PAGE. The specific protein was further purified via histidine tag using a nickel-NTA column. The protein was further dialyzed with phosphate-buffered saline (PBS) by using a dialysis membrane to remove excess urea.

Immunization, challenge, and protective efficacy

A total of ninety-six 5-week-old specific pathogen free (SPF) female BALB/c mice were randomly assigned into 12 groups (n = 8 per group), fasted for 20 h, and injected with the constructed vaccine candidates according to the scheme presented in Table 2. All mouse groups were challenged with 2×10^5 colony-forming unit (CFU)/200 μL of virulent *B. abortus* strain 544 at 30 days post-immunization via an IP route (Table 2). At day 15 post-challenge, the spleen was isolated from the mice, and the *B. abortus* strain 544 splenic bacterial load was determined as previously described [8]. The live *Salmonella* vector used in this study is cleared within 14 days post-immunization, and hence, it does not need differentiation from challenge strain 544. The recovery time 50 (RT50), the time in weeks in which half of the pulsed mice are estimated as fully recovered from immunization in the spleen, obtained for RB51 was approximately 3.8 weeks and, as a precaution, rifampin was added in the recovery media in order to differentiate RB51

Table 2. Immunization and challenge scheme

Group (n = 96)*	Strains and immunogen	Route	Formulation and dose (100 μL)	Wild-type challenge	Splenic *Brucella* recovery
A	Non-immunized control	IP	PBS	30 days post-immunization (2×10^5 CFU, IP)	15 days post-challenge
B	Vector control	IP	8×10^7		
C	RSrBL†	Oral	2×10^7 each (4 strains) + strain 544 LPS (10 μg)		
D	RSrBL	IM	2×10^7 each (4 strains) + strain 544 LPS (5 μg)		
E	RSrBL	SC	2×10^7 each (4 strains) + strain 544 LPS (5 μg)		
F	RSrBL	IP	2×10^7 each (4 strains)		
G	ST-SOD	IP	8×10^7		
H	ST-Omp	IP	8×10^7		
I	ST-BLS	IP	8×10^7		
J	ST-PrpA	IP	8×10^7		
K	LPS	IP	5 μg		
L	RB51	IP	8×10^7		

IP, intraperitoneal; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; IM, intramuscular; SC, subcutaneous; CFU, colony-forming unit; SOD, Cu-Zn superoxide dismutase; Omp, outer membrane protein; BLS, *Brucella* antigens lumazine synthase; PrpA, proline racemase subunit A; ST-SOD, rough *Salmonella* Typhimurium delivering *Brucella* SOD antigen; ST-Omp, rough *S.* Typhimurium delivering *Brucella* Omp19 antigen; ST-BLS, rough *S.* Typhimurium delivering *Brucella* BLS antigen; ST-PrpA, rough *S.* Typhimurium delivering *Brucella* PrpA antigen. *n = 8 per group· †Rough *Salmonella* delivering recombinant *Brucella* antigens and LPS formulation, four rough strains JOL1800 *Salmonella* live vectors—JOL1878, JOL1879, JOL1880, and JOL1881, each constitutively expressing *Brucella* immunogenic protein, *i.e.*, SOD, BLS, PrpA, and Omp19; combined with purified LPS isolated from strain 544.

from strain 544. *Brucella* cell counts of each group were $\log_{10}$ transformed and the protective index (PI) was calculated. PI is determined as the $\log_{10}$ CFU count of the test vaccine subtracted from the $\log_{10}$ CFU count of PBS.

Humoral immunity against whole-cell *Brucella* lysate and specific BLS antigen

In order to confirm that an immune response was successfully generated against the delivered antigen, the specific antibody produced during immunization with individual ST-recombinant *Brucella* strains was determined. Induction of antigen-specific antibodies in the cocktail of antigens was also assessed; for this experiment, the IP and oral routes of immunization were used. Serum samples were collected at weekly intervals for 4 weeks post-immunization. Briefly, 400 ng of purified *Brucella* antigen/well were used to coat ELISA wells (Microlon; Greiner Bio-One, Germany). Primary mouse serum was diluted 1:50 with PBS, and secondary horseradish peroxidase-conjugated goat anti-mice IgG antibodies were used at a 1:5,000 dilution. The colorimetric reaction was measured (TECAN, Austria) at

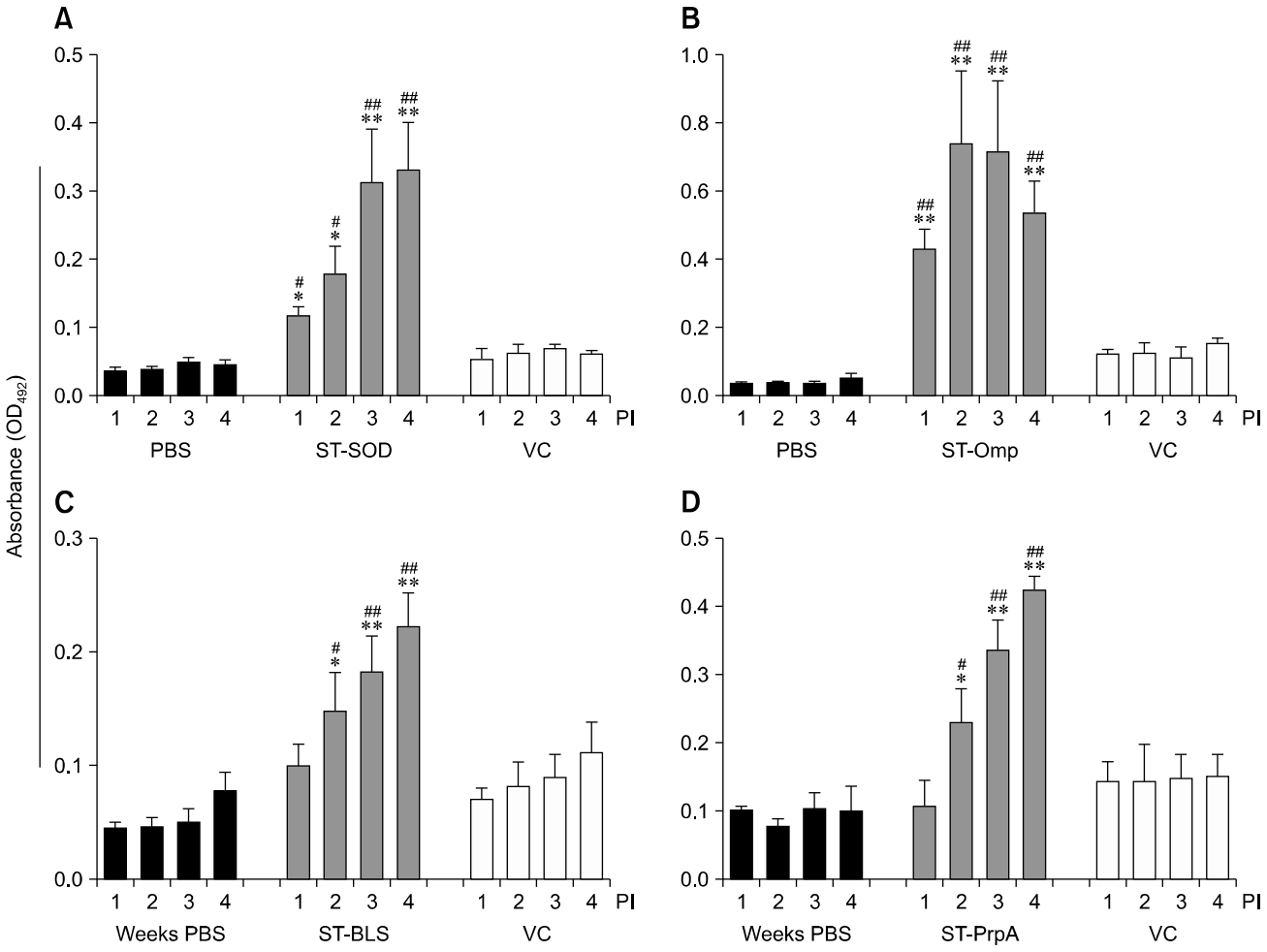

Fig. 1. Humoral immune responses induced by individual *Salmonella* Typhimurium (ST)-recombinant *Brucella* strains. The level of specific systemic immunoglobulin G (sIgG) production against the *Brucella* antigen was investigated by using indirect enzyme-linked immunosorbent assay. (A) The titers of sIgG specific to *Brucella* SOD of mice immunized with ST-SOD. (B) The titers of sIgG against *Brucella* Omp19 of mice immunized with ST-Omp. (C) The titers of sIgG against *Brucella* BLS of mice immunized with ST-BLS. (D) The titers of sIgG against *Brucella* PrpA of mice immunized with ST-PrpA. Antibody levels are expressed as mean $OD_{492} \pm SE$. OD_{492}, optical density at 492 nm; PBS, phosphate-buffered saline; SOD, Cu-Zn superoxide dismutase; VC, vector control; PI, post-immunization; Omp, outer membrane protein; BLS, *Brucella* antigens lumazine synthase; PrpA, proline racemase subunit A; ST-SOD, rough *S*. Typhimurium delivering *Brucella* SOD antigen; ST-Omp, rough *S*. Typhimurium delivering *Brucella* Omp19 antigen; ST-BLS, rough *S*. Typhimurium delivering *Brucella* BLS antigen; ST-PrpA, rough *S*. Typhimurium delivering *Brucella* PrpA antigen. Significant difference from the PBS control group, $^{*}p \leq 0.05$, $^{**}p \leq 0.005$. Significant difference from the VC group, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.005$.

492 nm (620 nm reference) after 10 min of post-development. The values for the binding of IgG to the respective antigens were expressed as the mean OD value ± SE.

Quantification of IL-17 and IL-23 cytokines

The influence of the route of administration of the cocktail vaccine on the selected cytokine levels was investigated. Relative cytokines levels were ascertained based on a protocol described previously with minor modifications [8]. Splenocytes were isolated from the immunized mice at 21 days post-immunization. Viable cells were placed in 24-well plates at a density of 2×10^6 cells/well. After 24 h of incubation at 37°C in a 5% CO_2 incubator, the splenocytes were pulsed with sonicated whole-cell *Brucella* 544 antigens (5 μg/well), followed by incubation for 16 h. Real-time PCR (RT-PCR) quantification was performed by using qPCR Master Mix (QuantiTect; Qiagen, Germany) to detect the levels of IL-17 and IL-23 cytokines. The reference gene β-actin was used as an endogenous control. Fold changes for the cytokines were calculated as the mean value of $2^{-\Delta\Delta CT}$ of a stimulated sample divided by the mean value of $2^{-\Delta\Delta CT}$ of an unstimulated sample [9].

Statistical analysis

Statistical analyses were performed where applicable. One-way analysis of variance (ANOVA) and Student's *t*-tests were used to determine statistically significant differences, with a p value $\leq$ 0.05 considered significant. Tukey's test was applied for *post hoc* analysis. Analyses were performed by using SPSS software (ver. 16.0; SPSS, USA).

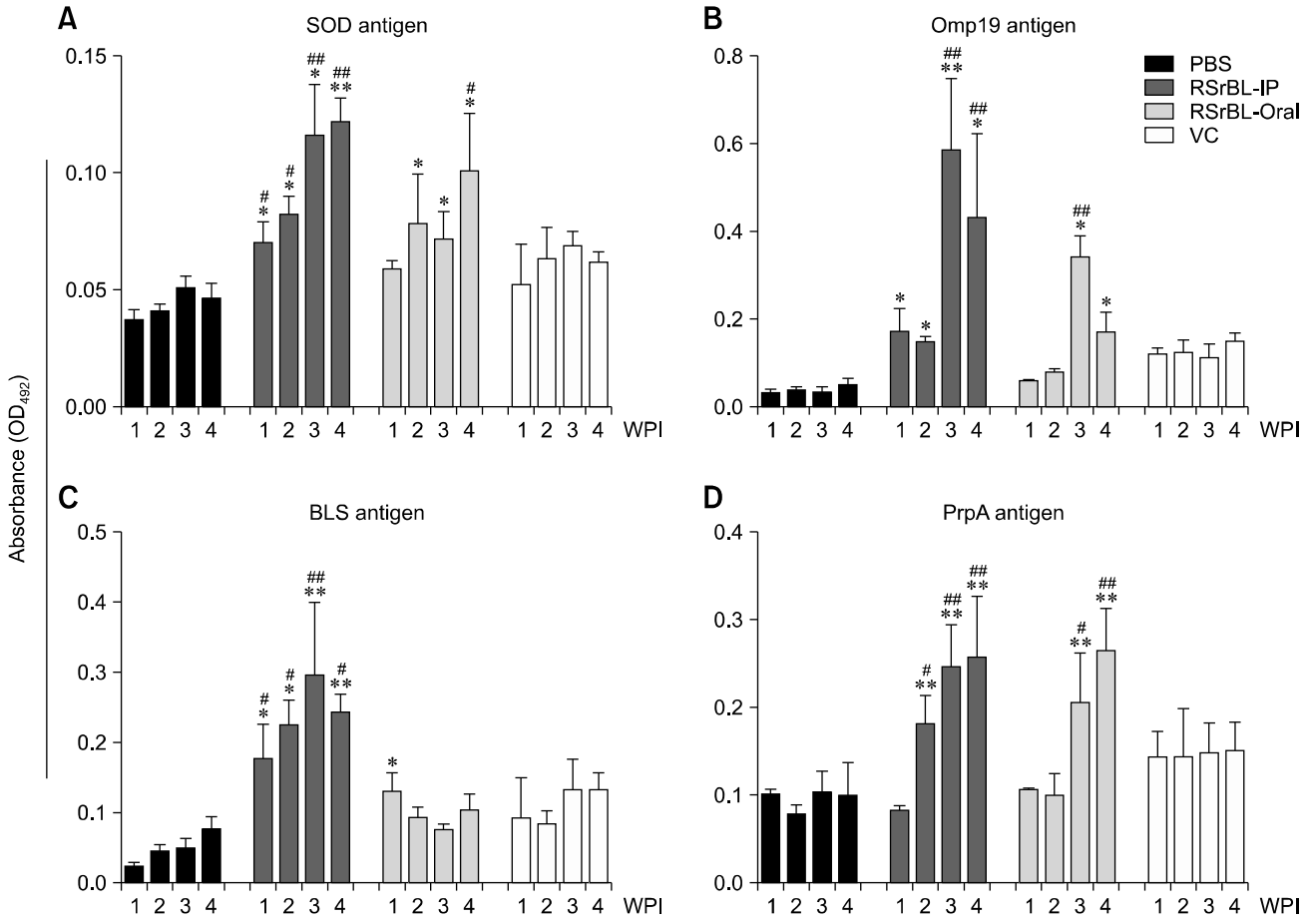

Fig. 2. The level of specific immunoglobulin G (IgG) against each *Brucella* antigen was investigated in mice immunized with a cocktail of *Salmonella typhimurium* (ST)-*Brucella* strains via the oral or IP routes. The titers of the systemic IgG response against *Brucella* SOD (A), *Brucella* Omp19 (B), *Brucella* BLS (C), and *Brucella* PrpA (D) were observed in mice immunized with ST-PrpA through the different routes. Antibody levels are expressed as mean OD_{492} ± SE. SOD, Cu-Zn superoxide dismutase; Omp19, outer membrane protein-19; BLS, *Brucella* antigens lumazine synthase; PrpA, proline racemase subunit A; PBS, phosphate-buffered saline; RSrBL, rough *Salmonella*-delivered recombinant *Brucella* antigens and lipopolysaccharide; IP, intraperitoneal; VC, vector control; WPI, weeks post-immunization. Significant difference from the PBS control group, $^*p \leq 0.05$, $^{**}p \leq 0.005$. Significant difference from the VC group, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.005$.

Results

Induction of humoral response against delivered antigens

Based on indirect ELISA, antibody production against the selected antigens was observed in mice immunized with the ST delivery strains. Anti-*Brucella* antigen IgG production was detectable at the 1st week post-immunization among certain individual mice (Fig. 1). At 3 weeks post-immunization, all individual vaccine deliveries induced significant differences in the immunized mice from those in the PBS and vector control groups ($p \leq 0.05$). Immunization with the ST-OmpA strain induced the highest overall production of IgG when compared to the other ST-strain-immunized groups.

The production of *Brucella* protein specific antibody was also determined in mice groups that were immunized with the complete rough *Salmonella*-delivered recombinant *Brucella* antigens and LPS (RSrBL) vaccine. Overall, mouse groups immunized via oral or IP routes showed production of specific IgG (Fig. 2). The maximum level of IgG production was observed against the Omp19 antigen. A significant increase in IgG production was observed as early as the 1st week post-immunization ($p \leq 0.05$). The fraction of anti-PrpA antibodies increased until the 4th week post-immunization (panel D in Fig. 2), while a declining pattern of IgG production was observed in the other groups. Compared to other antigens, there was weaker induction of the anti-SOD antibody (panel A in Fig. 2) when the SOD antigen was applied in combination with other antigens.

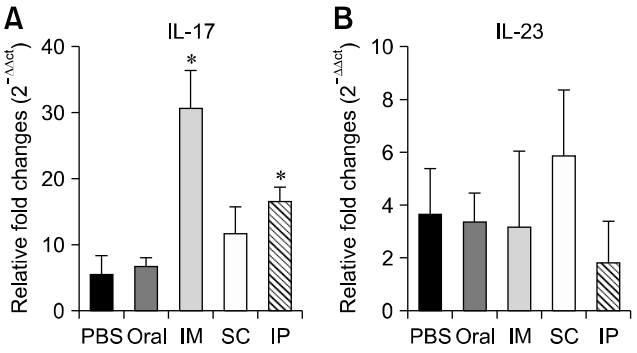

Fig. 3. Relative cytokine quantification. Fold change in expression of splenocyte cytokines upon *in vitro Brucella* sonicated lysate antigen stimulation was determined by quantitative polymerase chain reaction. Values represent relative expression of the cytokine normalized to the β-actin endogenous control. (A) Relative expression of the interleukin (IL)-17 cytokine. (B) Relative expression of the IL-23 cytokine. Relative cytokine levels are expressed as mean fold change ± SE. The asterisks indicate significant differences between cytokine expression of the immunized group and the non-immunized PBS group (*$p \leq 0.05$). PBS, phosphate-buffered saline; IM, intramuscular; SC, subcutaneous; IP, intraperitoneal.

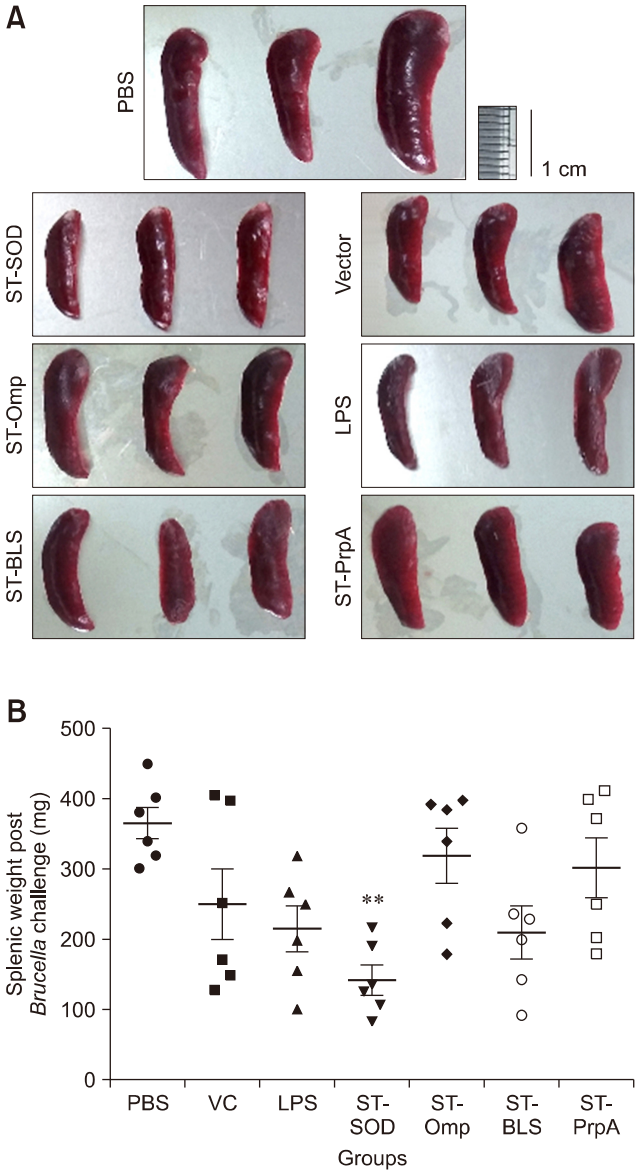

Fig. 4. Comparative splenic size and morphology. Representative spleen samples from each mouse group were aseptically isolated. (A) Appearance and relative size of spleens isolated from the mouse groups. (B) Relative differences in splenic weights of the mouse groups. Compared to spleens in the vaccinated groups, spleens of non-immunized mice were enlarged; however, there was no strong correlation between splenic weight and vaccine protection as non-enlarged spleens also harbor a considerable load of challenge bacteria. PBS, phosphate-buffered saline; ST-SOD, rough *Salmonella* Typhimurium delivering *Brucella* SOD antigen; ST-Omp, rough *S.* Typhimurium delivering *Brucella* Omp19 antigen; ST-BLS, rough *S.* Typhimurium delivering *Brucella* BLS antigen; LPS, lipopolysaccharide; ST-PrpA, rough *S.* Typhimurium delivering *Brucella* PrpA antigen; VC, vector control. **$p \leq 0.005$.

Relative cytokine levels

The induction of IL-17 and IL-23 cytokines by different routes of administration of the cocktail vaccine was investigated. The level of selected cytokines was determined on extracted RNA of *in vitro* whole-cell lysate *Brucella* antigen-stimulated splenocytes (Fig. 3). The pro-inflammatory cytokine IL-17 levels were determined for each mouse group (panel A in Fig. 3). The IM and IP routes of immunization exhibited significant increases in IL-17 levels compared the level in PBS non-immunized mice. The level of another key mediator of inflammation, cytokine IL-23, was also determined (panel B in Fig. 3). The SC immunization route produced increases in IL-23 levels; however, the change overlapped with that in the PBS control and the difference was not statistically significant.

Protection efficacy of individual ST vaccine strain

The protective efficacy conferred by individual strains was determined in a mouse model. SPF-BALB/c mice (4 weeks old) were immunized with the constructed vaccine candidates and then injected with virulent *B. abortus* 544 at day 30 post-immunization. Mice were sacrificed at day 15 post-challenge. The relative difference in splenic weight was measured in each of the mouse groups (Fig. 4). Spleens of non-immunized mice were enlarged when compared to those in the vaccinated groups. However, there was no strong correlation between splenic weight and challenge protection, as the non-enlarged spleens also harbored considerable loads of challenge bacteria. The degree of challenge-bacteria burden in the target organ reflected the efficacy of immunization. In comparison with PBS results, all individual immunization strains, except the ST-PrpA strain, conferred significant levels of protection ($p \leq 0.05$; panel A in Fig. 5). In the current experimental setting, maximum protection was conferred by the ST-SOD strain followed by that of LPS, ST-BLS, and ST-Omp. The protective indices were determined to be 0.51, 0.99, 0.22, 0.42, and 0.01 for LPS, ST-SOD, ST-Omp, ST-BLS, and ST-PrpA, respectively.

Effect of route of immunization on protective efficacy of the combined LPS-*Brucella* vaccine

The influence of immunization routes was investigated. The lowest number of challenge strain CFUs was recovered from mice immunized via the IP route (panel B in Fig. 5). This means the maximum level of protection was conferred to mice when the formulation was administered via the IP route. However, the difference between the IP and IM routes was not significant. In addition, the SC immunization route also conferred a range of protection similar to that of the IP and IM routes. The difference between SC and IM routes was not significant, but there was a significant difference between the SC and IP routes. Oral immunization produced the lowest level of protection among the compared routes; however, the level was significantly different from mice immunized via the IP route with vector only

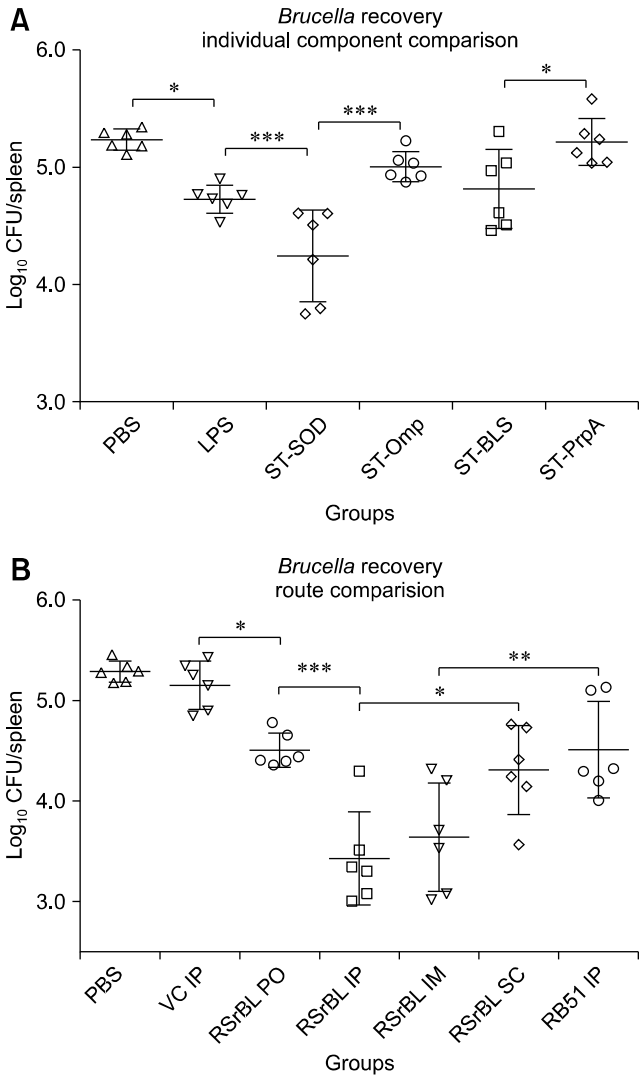

Fig. 5. Splenic bacterial recovery. The colony-forming unit (CFU) level of the challenge strain *Brucella abortus* 544 was determined. The reduction in the splenic CFU count is correlated with protection. (A) Bacterial recovery after immunization with individual *Salmonella* Typhimurium (ST)-*Brucella* strains. Mice immunized with the ST-SOD strain had the lowest bacterial count, which indicates ST-SOD provided the highest level of protection. All individual immunization components, except ST-PrpA, showed a significant level ($p \leq 0.05$) of protection. (B) Immunization route comparison. The lowest bacterial count was observed in mice immunized via the IP route, followed by the IM, SC, and oral routes. The data are represented as $\log_{10}$ CFU/spleen. PBS, phosphate-buffered saline; LPS, lipopolysaccharide; ST-SOD, rough *S.* Typhimurium delivering *Brucella* SOD antigen; ST-Omp, rough *S.* Typhimurium delivering *Brucella* Omp19 antigen; ST-BLS, rough *S.* Typhimurium delivering *Brucella* BLS antigen; ST-PrpA: rough *S.* Typhimurium delivering *Brucella* PrpA antigen; VC, vector control; IP, intraperitoneal; RSrBL, rough *Salmonella*-delivered recombinant *Brucella* antigens and lipopolysaccharide; IM, intramuscular; SC, subcutaneous. Significant difference between indicated groups, $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$.

(control). Interestingly, the immunization with the combined LPS-*Brucella* vaccine via an IP or IM route produced significantly higher levels of protection than that from the inoculation with commercial strain RB51 vaccine via an IP route ($p \leq 0.005$). The protective indices were determined to be 0.07, 0.72, 1.81, 1.59, 0.92, and 0.72 for vector control-IP, RSrBL-Oral, RSrBL-IP, RSrBL-IM, RSrBL-SC, and RB51-IP, respectively.

Discussion

In this study, we evaluated the humoral immunogenicity and protective efficacy of *Brucella* vaccine candidates, singly or in combination; an evaluation that was necessary for dose optimization and formulation of a more efficient *Brucella* vaccine. Immunogenicity of a vaccine candidate or vaccine lot-testing of *B. abortus* S19 is usually carried out in a mouse model [5]. The level of protection index observed in this model is similar to those showed in bovine vaccination. However, testing of vaccine candidates in the final host may be necessary to prove the efficacy of the conserved proteins. In order to confirm the successful delivery of each antigen by the delivering vector, specific antibodies need to be evaluated. Also, the role of the humoral response in protection against *Brucella* infection should not be overlooked, although the replication niche of the pathogens is intracellular [22]. Complement killing and opsonization may also have an important role in reducing the bacterial load before the organism becomes intracellular [2]. In this study, successful delivery of each antigen was confirmed by performing indirect ELISA. Among the candidate strains, the ST-Omp strain induced the highest level of IgG antibodies in mouse serum; counter-intuitively, the level of anti-PrpA antibodies was much lower (Fig. 1). The specific antibody production levels of the other tested strains were also determined. In the weeks following the immunization of the cocktail vaccine, the level of antibody production against each antigen was determined by using the appropriate recombinant protein. Antibody production against each antigen in this cocktail formulation was less than that from the individual strain vaccinations.

The effect of the combined *Brucella* vaccine on the induction of pro-inflammatory cytokines IL-17 and IL-23 was also investigated. The IL-17 cytokine stimulates the generation of inflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6, as well as inflammatory chemokines that facilitate the recruitment of neutrophils and macrophages [11]. IL-17 also promotes expression of acute-phase response genes and antimicrobial substances. It is also implicated in host-resistance against fungal agents, such as *Candida albicans* [19], and bacterial pathogens [23]. Contradictory results have been observed in host resistance to brucellosis, in which it was demonstrated that Th-17 cells were dispensable for the control of *Brucella melitensis* infection in mice [21]. On the other hand, IL-17 was implicated in protection against nasal infection of *Brucella* bacteria via potentiation of interferon-γ [17]. In this study, we examined the relationship between route of administration of the RSrBL vaccine and subsequent IL-17 and IL-23 production. Mice immunized via the IM and IP routes showed significant increases in IL-17 production, whereas such increases were not observed following immunization via the oral and SC routes. The mechanism involved in producing these different results is unclear. The levels of IL-23 were also determined (panel B in Fig. 3). IL-23 has a pivotal role in maintaining the population of Th-17 cells, which are a novel T-cell subset involved in the antimicrobial immune response and the development of autoimmune diseases [10]. IL-23 activates STAT3, resulting in a direct binding of phosphorylated STAT3 to IL-17 or IL-17F promoters [1]. No significant induction of the IL-23 cytokine upon *Brucella* antigenic stimulation was observed.

As anticipated, we observed significant levels of protection when the vaccine strains are used singly. However, none of the single strains could surpass the immunization protection when the strains were used in combination. While it can be attributed to an inclusion of LPS in our previous study [8], in this study, we determined that a cocktail of vaccine strains without LPS could only confer protection in the range of 0.3 to 0.5. Moreover, the protection level was in the range of 0.2 to 0.5 when only LPS was used. In addition, the critical role of each protein in a natural infection was not tested in the current experimental setting. For instance, the PrpA protein was included to reduce the duration of chronicity. The shorter period of study, or even the mouse model used, may not provide an accurate representation of the disease in its chronic phase. Hence, whether any of the strain is dispensable from the final combination, may require further testing and trials.

In the comparison of the routes of administration, the IP route showed the highest level of protection. This advantage of the IP route may be attributed to the direct interaction with the antigen-presenting cells (APCs) and the bacterial vector. However, this route of immunization may be impractical for field deployment, as it requires a well-trained person for effective IP delivery, especially in a large animal host. Although the level of protection exhibited by mice immunized via the IM route was lower than that via the IP route, the difference was not significant. Immunization via the oral route produced the lowest levels of protection among the tested routes. This result may be attributed to the presence of degrading enzymes, low stomach pH, and the presence of food in the digestive tracts, which can reduce the effectiveness of processing by APCs. Hence, optimization of vaccine formulation may be necessary to improve the protective efficacy of an oral vaccine. For example, when administered via an oral route, the vaccine could be protected from the acidic pH of the stomach by using bicarbonate salts, or from enteric proteolytic enzymes by using and enteric-coating system.

Taken together, the present study revealed that a certain level

of protection was conferred by selected individual vectored antigens, which indicates that ST-delivered *Brucella* antigens can successfully induce an antigen-specific immune response. It was also demonstrated that the efficacy of the vaccine candidate varied according to the route of administration. The IM route, which can reduce local inflammatory reactions, may be preferred to the SC or IP route due to its relative ease of administration. Although immunization via an oral administration showed an insufficient level of protection against the challenge, formulation improvements may be considered for application in human anti-*Brucella* vaccines; however, further study is needed.

Acknowledgments

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant No. HI16C2130).

Conflict of Interest

The authors declare no conflicts of interest.

References

1. **Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, Yoshimura A, Hennighausen L, O'Shea JJ.** Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc Natl Acad Sci U S A 2006, **103**, 8137-8142.
2. **Corbeil LB, Blau K, Inzana TJ, Nielsen KH, Jacobson RH, Corbeil RR, Winter AJ.** Killing of *Brucella abortus* by bovine serum. Infect Immun 1988, **56**, 3251-3261.
3. **Corbel MJ.** Brucellosis in Humans and Animals. World Health Organization, Geneva, 2006.
4. **Han Y, Han X, Wang S, Meng Q, Zhang Y, Ding C, Yu S.** The *waaL* gene is involved in lipopolysaccharide synthesis and plays a role on the bacterial pathogenesis of avian pathogenic *Escherichia coli*. Vet Microbiol 2014, **172**, 486-491.
5. **International Office of Epizootics (OIE).** Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds, and Bees. 7th ed. OIE, Paris, 2009.
6. **Kim WK, Moon JY, Kim S, Hur J.** Comparison between immunization routes of live attenuated *Salmonella* Typhimurium strains expressing BCSP, Omp3b, and SOD of *Brucella abortus* in murine model. Front Microbiol 2016, **7**, 550.
7. **Lalsiamthara J, Kamble NM, Lee JH.** A live attenuated *Salmonella* Enteritidis secreting detoxified heat labile toxin enhances mucosal immunity and confers protection against wild-type challenge in chickens. Vet Res 2016, **47**, 60.
8. **Lalsiamthara J, Lee JH.** *Brucella* lipopolysaccharide reinforced *Salmonella* delivering *Brucella* immunogens protects mice against virulent challenge. Vet Microbiol 2017, **205**, 84-91.
9. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001, **25**, 402-408.
10. **McGeachy MJ, Cua DJ.** Th17 cell differentiation: the long and winding road. Immunity 2008, **28**, 445-453.
11. **Mills KH.** Induction, function and regulation of IL-17-producing T cells. Eur J Immunol 2008, **38**, 2636-2649.
12. **Nymo IH, Tryland M, Godfroid J.** A review of *Brucella* infection in marine mammals, with special emphasis on *Brucella pinnipedialis* in the hooded seal (*Cystophora cristata*). Vet Res 2011, **42**, 93.
13. **Oñate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H.** Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infect Immun 1999, **67**, 986-988.
14. **Pappas G, Akritidis N, Bosilkovski M, Tsianos E.** Brucellosis. N Engl J Med 2005, **352**, 2325-2336.
15. **Pasquevich KA, Ibañez AE, Coria LM, García Samartino C, Estein SM, Zwerdling A, Barrionuevo P, Oliveira FS, Seither C, Warzecha H, Oliveira SC, Giambartolomei GH, Cassataro J.** An oral vaccine based on U-Omp19 induces protection against *B. abortus* mucosal challenge by inducing an adaptive IL-17 immune response in mice. PLoS One 2011, **6**, e16203.
16. **Schurig GG, Roop RM 2nd, Bagchi T, Boyle S, Buhrman D, Sriranganathan N.** Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 1991, **28**, 171-188.
17. **Skyberg JA, Thornburg T, Pascual DW.** IL-17 is required for protective immunity to nasal *Brucella* infections in an IFN-γ-dependent fashion. J Immunol 2009, **182** (1 Suppl), 39.23.
18. **Spera JM, Ugalde JE, Mucci J, Comerci DJ, Ugalde RA.** A B lymphocyte mitogen is a *Brucella abortus* virulence factor required for persistent infection. Proc Natl Acad Sci U S A 2006, **103**, 16514-16519.
19. **Trautwein-Weidner K, Gladiator A, Nur S, Diethelm P, LeibundGut-Landmann S.** IL-17-mediated antifungal defense in the oral mucosa is independent of neutrophils. Mucosal Immunol 2015, **8**, 221-231.
20. **Velikovsky CA, Cassataro J, Giambartolomei GH, Goldbaum FA, Estein S, Bowden RA, Bruno L, Fossati CA, Spitz M.** A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun 2002, **70**, 2507-2511.
21. **Vitry MA, De Trez C, Goriely S, Dumoutier L, Akira S, Ryffel B, Carlier Y, Letesson JJ, Muraille E.** Crucial role of gamma interferon-producing $CD4^+$ Th1 cells but dispensable function of $CD8^+$ T cell, B cell, Th, and Th17 responses in the control of *Brucella melitensis* infection in mice. Infect Immun 2012, **80**, 4271-4280.
22. **Vitry MA, Hanot Mambres D, De Trez C, Akira S, Ryffel B, Letesson JJ, Muraille E.** Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 2014, **192**, 3740-3752.
23. **Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J,**

Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001, **194**, 519-527.