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Intranasally administered anti-*Brucella* subunit vaccine formulation induces protective immune responses against nasal *Brucella* challenge

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ABSTRACT

The present study was aimed to develop a safe and effective anti-*Brucella* subunit vaccine for mucosal protection against the respiratory exposure of *Brucella* infection. A chitosan-based *Brucella* nasal vaccine (BNV) was formulated using well-known *Brucella* immunogens, sodC, omp19, BLS and PrpA and tested against nasal *Brucella* challenge in BALB/c mice. The mice were intra-nasally vaccinated with sterile phosphate buffer saline (PBS), BNV or BNV plus *Brucella* LPS, and humoral (systemic IgG and mucosal IgA) and cell-mediated immune responses were analyzed. Results showed that mice vaccinated with either BNV or BNV plus LPS elicited significantly ($p < 0.05$) high IgG and IgA responses compared to the PBS control. The IgG responses were significantly ($p < 0.05$) higher than IgA levels, which showed almost comparable levels observed in either intestines or in lungs. Furthermore, the IgG and IgA responses against each individual component of the BNV formulation indicated that omp19 induced highest levels of both IgG and IgA levels than the other constituents of BNV formulation. Upon re-stimulation of the splenocytes with *Brucella* whole cell lysate, significantly ($p < 0.05$) high IFN-γ levels, lymphocyte proliferation, and $CD4^+$ T cell responses were observed in mice vaccinated with BNV or BNV plus LPS. Upon sub-lethal nasal challenge with wild-type *Brucella strain*, vaccinated mice showed significant reduction of *Brucella* recovery in lungs and spleen compared to the PBS control. This study indicates that BNV formulation with or without *Brucella* LPS efficiently induced humoral and cell-mediated immune responses and conferred significant protection against the sub-lethal *Brucella* challenge.

1. Introduction

Brucella infection remains as a significant zoonotic threat throughout the world. It is caused by a group of Gram-negative bacteria of the genus *Brucella* (Yang et al., 2013). The bacterium can be easily disseminated by ingestion, inhalation or contact exposure (Surendran et al., 2013) and is considered as a category B bioterrorism agent by the Center for Disease Control in the United States ("Emergency Preparedness and Response," 2018). Lack of human vaccines demonstrates the vulnerability of human population and, therefore, warrants intensive research investigations into development of an effective and safe vaccines against human brucellosis. Currently available live attenuated *Brucella abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev-1 vaccine strains are licensed to control livestock brucellosis (Lalsiamthara and Lee, 2017). However, residual virulence associated with these vaccine strains have not been completely eliminated (Godfroid et al., 2011). Due to the virulence of livestock *Brucella* vaccines on humans, the professionals dealing with animal vaccination and veterinary care are particularly vulnerable for the disease. These facts emphasize the need for alternative safe vaccines that can be equally potent on animals and particularly for humans for the control of brucellosis. It is a fact that *Brucella* species can infect epithelial cells allowing the entry via mucosal surfaces (Bhattacharjee et al., 2006; Poester et al., 2013; Clapp et al., 2016), moreover, *Brucella* species are highly infection through aerosol route (Doganay and Doganay, 2013). Therefore, a vaccine which could elicit mucosal immunity especially in the nasopharyngeal region would be possibly the ideal way to contain *Brucella* infections in place of acquisition. To accomplish this strategy, in the present study, we have chosen nasal route of administration to deliver vaccine antigens. Further to enhance the safety of the vaccine more towards human use, we used *Brucella* subunit proteins which could be substantially safer than currently available live forms of *Brucella* vaccines. In order to formulate an anti-*Brucella* nasal vaccine (BNV), four highly conserved *Brucella* antigens, namely Cu-Zn superoxide dismutase (sodC), outer membrane protein 19 (omp19), lumazine synthase (BLS) and proline racemase subunit A (PrpA) were selected as

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these antigens have been reported for their significant role in conferring protection against *Brucella* infection in mice model (Muñoz-Montesino et al., 2004; Cassataro et al., 2007; Lalsiamthara and Lee, 2017). Among these antigens, sodC and BLS reported to elicit mixed type of Th1 and Th2 immune responses (Velikovsky et al., 2002; Muñoz-Montesino et al., 2004) while as omp19 induces IL-17 cytokine production, which (Pasquevich et al., 2011) is essential in mucosal immunity. Furthermore, PrpA antigen is a potent B cell stimulant and thus be important in humoral immunity (Lalsiamthara and Lee, 2017). We hypothesize that intranasal administration of a cocktail of sodC, omp19, BLS and PrpA may efficiently stimulate both Th1 and Th2 type of immunities and provide subsequent protection against the respiratory exposure of *Brucella* infection. Several considerable studies have demonstrated that *Brucella* lipopolysaccharide (LPS) alone protects mice against wild-type *Brucella* challenge (Bhattacharjee et al., 2006), and, therefore, to make our vaccine more efficient, we included LPS, extracted from a virulent *B. abortus* 544 strain, as a supplement in the formulation. The co-mix of sodC, omp19, BLS, PrpA and LPS was intranasally delivered via chitosan nanoparticles in order to enhance retention in the nasal mucosa. Positively charged chitosan nanoparticles are known to form nanoclusters with protein antigens that may enhance antigen targeting to antigen presenting cells for efficient elicitation of antigen-specific T and B cell immune responses (Carroll et al., 2016).

Herein, we show that intranasal co-administration of sodC, omp19, BLS, PrpA, and LPS antigens coupled with chitosan nanoparticles elicited efficient antigen-specific humoral and cell-mediated immune responses, and significantly protected mice against the virulent *B. abrotus*544 nasal challenge.

2. Materials and methods

2.1. Bacterial strains, primers, plasmids and media

The bacterial strains, plasmids, and primers used in this experiment are listed in supplementary Table1. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth supplemented with antibiotic Kanamycin (50 μg/ml). *B. abortus* 544 strain was grown in *Brucella* medium (BD, USA) at 37 °C in a 5% CO_2 atmosphere without any addition of antibiotics. Appropriate biosafety measures were enforced while handling *B. abortus* biosafety level 3 microorganism.

2.1.1. Cloning and purification of Brucella immunogenic proteins

Open reading frames encoding sodC, omp19, BLS and PrpA of *B. abortus* 544 strain were PCR amplified and cloned into pET28a (+) vector system (Novagen, Madison, WI, USA), respectively. Recombinant plasmids were subsequently transformed into *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) for protein expression as previously described (Hajam et al., 2013). The soluble protein fractions were purified by Ni-NTA (Qiagen, Valencia, CA, and USA) affinity column chromatography (Poly-Prep, Bio-Rad, Hercules, CA, USA) and the purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each protein fraction was buffer exchanged to phosphate buffered saline (PBS) using buffer exchange columns (GE Healthcare, UK) and concentrated using protein concentrator columns with 10 kDa cutoff limit (Amicon Ultra 4, Millipore, Ireland) before being used in the vaccine formulation. Protein concentration was measured by Bradford method (Bradford, 1976). The purified proteins were then stored at −80 °C until further use.

2.2. Vaccine formulation

Lipopolysaccharides from virulent *B. abortus* 544 strain was extracted and purified using phenol-based commercial LPS extraction kit (iNtRON Biotechnology, South Korea) according to manufacturer's instructions. The concentration of purified LPS was determined by dry weight measurement and resuspended in 10 mM Tris−HCl, pH 8.0). Five micrograms of purified *Brucella* LPS was directly mixed with 25 μg of each sodC, omp19, BLS, and PrpA proteins in 0.1% w/v concentration chitosan (Sigma, St. Louise, USA) in order to formulate a nasal dose. Twenty microliters of the formulation was assigned as a dose per mice. The vaccine formulation was kept at 4 °C for 2 h for equilibration and interact with chitosan nanoparticles before administration in mice.

2.3. Immunization and challenge studies

Animal experiments were approved (CBNU2015-0085) 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. All mice used in this study were housed and maintained humanely. 5-week old specific-pathogen-free female BALB/c mice were randomly divided into three groups (n = 16 in each group) and intra-nasally vaccinated with PBS, BNV formulation plus LPS or BNV formulation alone in a volume of 20 μl per mice. A booster vaccination was carried out on the 14th day post-primary vaccination. Blood collection from the retro-orbital plexus (Parasuraman et al., 2010) and plasma serum preparation (N = 8) (Tuck et al., 2009) were conducted at 0, 12 and 26 days post-primary immunization for IgG analysis. Four mice from each group were euthanized at either 14^{th} or 28th day post-primary immunization to collect intestinal or lung wash samples for IgA estimation. Briefly, thoracic cavity was aseptically opened to expose lungs. Then 2 ml of PBS directly injected to inflate lungs. Lung washes were collected by back and forth injection of PBS. Approximately 1 ml volume of lung washes was stored at −20 °C for analysis. To collect intestinal washes, the total intestine was aseptically removed into a Petri dish. Total volume of 1 ml PBS was injected using a syringe needle by flushing the intestinal walls from the distal end on the intestine. Approximately 1 ml volume was stored at −20 °C for analysis. Furthermore, splenocytes from four mice from each group were harvested on the 14th-day post-primary immunization and used for the analysis of lymphocyte proliferation assay by MTT-based assay, flow cytometry, and mRNA IFN-γ induction by qRT-PCR assay. At 30th day post-primary immunization, remaining mice (N = 8) were intranasally challenged with 2×10^4 colony forming units (CFU) of a virulent *B. abortus* 544 strain. Fourteen days post-challenge, four mice were euthanized and *B. abortus* 544 bacterial load were determined in whole lungs and spleen as described previously (Lalsiamthara and Lee, 2017). Moreover, lungs and spleen of remaining mice were aseptically collected and subjected to histopathological analysis by eosin and hematoxylin staining procedure as previously described (Kim et al., 2018).

2.4. Antigen-specific ELISA

To determine the level of anti-*Brucella* antibody production in response to vaccination, humoral responses of control and vaccinated mice were investigated in an indirect ELISA (Lalsiamthara and Lee, 2017). The IgG (n = 8) and IgA (n = 4) antibody levels were measured against either *Brucella* whole cell lysate (300 ng/well) or individual protein antigens (300 ng/well) of BNV plus LPS formulation using the splenocytes harvested from BNV plus LPS treated mice.

2.5. IFN-γ cytokine responses in vaccinated mice

Antigen-specific induction of IFN-γ was measured at mRNA transcription level using quantitative real-time PCR (qRT-PCR) assay. Splenocytes (N = 4) were harvested from vaccinated and control mice at 14th day post-primary immunization (Park et al., 2018) and 1×10^6 cells/well were stimulated with *Brucella* whole cell lysate antigen (10 μg/ml) for 24 h at 37 °C in 5% CO_2. Post-stimulation, total RNA was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The cDNA was synthesized from an equal quantity of 1 μg of RNA using SuperScript III Reverse Transcriptase kit

(Invitrogen, San Diego, CA) following manufacturer's procedure and stored at −20 °C until use. The qRT-PCR for the IFN- γ induction was performed in ABI applied biosystems using SYBR Green PCR master mix as described previously (Won and Lee, 2016). The relative amounts of cytokine mRNA present were determined by $2^{-\Delta\Delta}$CT method (Pfaffl, 2001).

2.6. Splenocyte proliferation assay

Two weeks post-primary immunization, in vitro proliferation of splenocytes isolated from vaccinated and PBS control mice (n = 4) were evaluated. Ten thousand cells per well were re-stimulated with *Brucella* whole cell lysate or individual protein antigens (10 μg/ml) of BNV plus LPS formulation and incubated for 72 h at 37 °C in a 5% CO_2 incubator. For the latter, splenocytes harvested from BNV plus LPS treated mice were used. Cell proliferative response was evaluated by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide]-based assay as described previously (Dar et al., 2013).

2.7. FACS analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations

Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell populations were carried out on mice splenocytes (n = 4) harvested at 14th day post-primary immunization as previously described (Lalsiamthara and Lee, 2017). Briefly, 2×10^5 viable cells were stimulated with *Brucella* whole cell lysate antigen (10 μg/ml) or RPMI media alone for 24 h at 37 °C in a 5% CO_2 atmosphere and then the cells were harvested and stained with PE-labeled anti-CD3e, PerCPVio700-labeled anti-CD4, and FITC-labeled anti-CD8a monoclonal antibodies as previously described (Won et al., 2017). The $CD3^+$ T cell population was gated to analyze the $CD3^+CD4^+$ and $CD3^+CD8^+$ subpopulations in vaccinated and control mice groups.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 6.00 program (San Diego, CA, USA.) One way analysis of variance (ANOVA) with Tukey`s multiple comparison test were conducted to determine the statistical difference among vaccinated and control groups. P value < 0.05 were considered statistically significant.

3. Results

3.1. Purification and confirmation of Brucella antigens

The *Brucella* antigens, namely sodC, omp19, BLS and PrpA were successfully cloned and expressed in BL21 (DE3) host strain. The expressed proteins were purified by Ni-NTA affinity column chromatography, and the purity and size of *Brucella* proteins were confirmed by SDS-PAGE analysis. The SDS-PAGE analysis revealed that purity of expressed protein well above 95% as single expected band of protein size was observed (Supplementary Fig. 1).

3.2. Humoral responses against Brucella whole cell lysate and individual antigens

The ability of the BNV formulation to induce systemic and mucosal antibody responses was evaluated in mice that had been nasally immunized with PBS or BNV formulation with and without LPS supplement. Both mice groups immunized with BNV formulation with or without LPS have generated significantly high IgG levels compared to PBS control as measured on 12th and 26th day post immunization ($P < 0.05$) (Fig. 1A). A significant increase in the IgG antibody levels due to addition of LPS into BNV formulation was not evident by 26th day post-immunization (Fig. 1A). A significantly high IgA antibody response was observed in BNV immunized mice on 26th day post immunization compared to the PBS control in both lung and intestinal wash samples ($P < 0.05$). However, IgA responses of BNV formulation with and without LPS were comparable and did not make any significant increase in both lung and intestinal washes (Fig. 2A and C). Next, we measured IgG and IgA responses elicited against each individual component of the BNV plus LPS formulation. Serum samples drawn on 26th day post-primary vaccination from BNV plus LPS treated mice showed that sodC and omp19 antigens elicited significantly ($p < 0.05$) higher IgG responses compared to BLS and PrpA antigens, which induced almost comparable levels (Fig. 1B). The LPS antigen-induced least IgG responses that were though significant ($p < 0.05$) compared to the PBS control group (Fig. 1B). To measure mucosal IgA responses, 4 mice in each group were sacrificed at 14th and 28th day post-primary immunization and intestinal and lung wash samples were collected (Fig. 2A and C). Our results in lung washes showed that highest IgA responses were induced against omp19 and BLS while sodC, PrpA, and LPS showed almost comparable levels, but statistically significant ($p < 0.05$) than PBS control group (Fig. 2B). The highest IgA responses in intestinal wash samples were generated by Omp19, BLS and PrpA that were comparable to each other. The responses induced by sodC and LPS were lower to rest of the antigens however statistically significant than the PBS control group (Fig. 2D). All these results clearly indicate that intra-nasally delivered BNV formulation can induce both systemic and mucosal antibody responses and addition of LPS has no significant effect on the augmentation of antibody formation.

3.3. Cell mediated immune responses

Secretion of cytokines is an indication of the activation of cell-mediated immune responses. It was shown that, IFN- γ secretion and Th1 type immune response is crucial for intracellular *Brucella* clearance. The effectiveness of BNV formulation was therefore evaluated in immunized mice by considering IFN- γ as a signature cytokine for Th1 type immune response and quantified by qRT-PCR (Fig. 3). For this, on 14^{th} day post-immunization, splenocytes from 4 mice were harvested and stimulated with *Brucella* lysate for 24 h under in-vitro conditions. A marked increase in IFN- γ response were observed in both groups immunized with BNV formulation with and without LPS addition as compared to the PBS control ($p < 0.05$). The cellular immune responses were further investigated by MTT-based splenocyte proliferation assay performed on 14th day post-primary immunization (Fig. 4A). Four mice from each immunized and control group were harvested and stimulated with whole *Brucella* cell lysate and incubated for three days. A significantly ($p < 0.05$) higher, but comparable proliferative responses were observed in mice immunized with either BNV formulation with or without LPS as compared to the PBS control group (Fig. 4A). We further measured lymphocyte proliferative responses against each component of BNV plus LPS formulation by stimulating with each antigen or with PBS as a control. Our results indicated that lymphocyte proliferative responses were significantly ($p < 0.05$) higher against each protein antigen compared to the LPS component (Fig. 4B).

Next, we investigated the $CD4^+$ and $CD8^+$ T cell proliferative responses to the *Brucella* whole cell lysate. The fraction of $CD3^+CD4^+$ T-cell subpopulation was significantly ($p < 0.05$) higher in mice vaccinated with either BNV plus LPS or BNV formulation alone as compared to the PBS control group (Fig. 5). The flow cytometry data showed approximately two-fold increases in $CD4^+$ T cell subpopulation in both vaccinated mice groups compared to the PBS control group (Fig. 5). Although, both the vaccinated mice groups showed higher $CD8^+$ T cell responses compared to the PBS control group, but the responses were non-significant. All these results suggest that our BNV vaccination can elicit both humoral and cell-mediated immune responses in mice model.

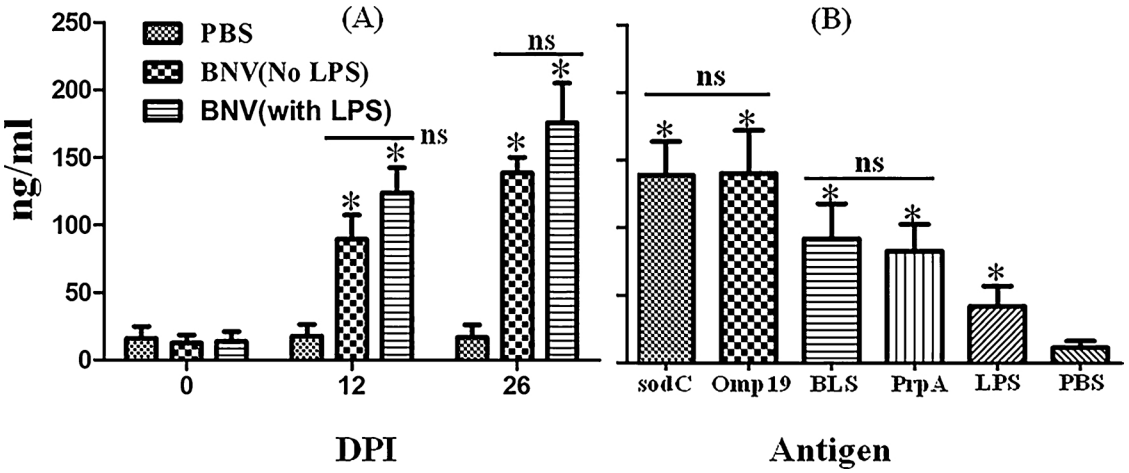

Fig. 1. Systemic IgG responses.

Mice (n = 16) were intranasally vaccinated with PBS, BNV or BNV plus LPS formulation and serum samples were collected at 0, 12, and 26 days post-primary immunization for IgG analysis by an indirect ELISA against *Brucella* whole cell lysate (A). IgG responses against each individual component of the BNV plus LPS formulation were performed on serum samples collected at 26th day post-immunization from BNV plus LPS treated mice (B). Each data points represent mean ± standard deviation (SD) of 8 animals (SD). * Indicates significant difference compared to PBS control; $p < 0.05$, ns; non-significant, DPI; days post-immunization.

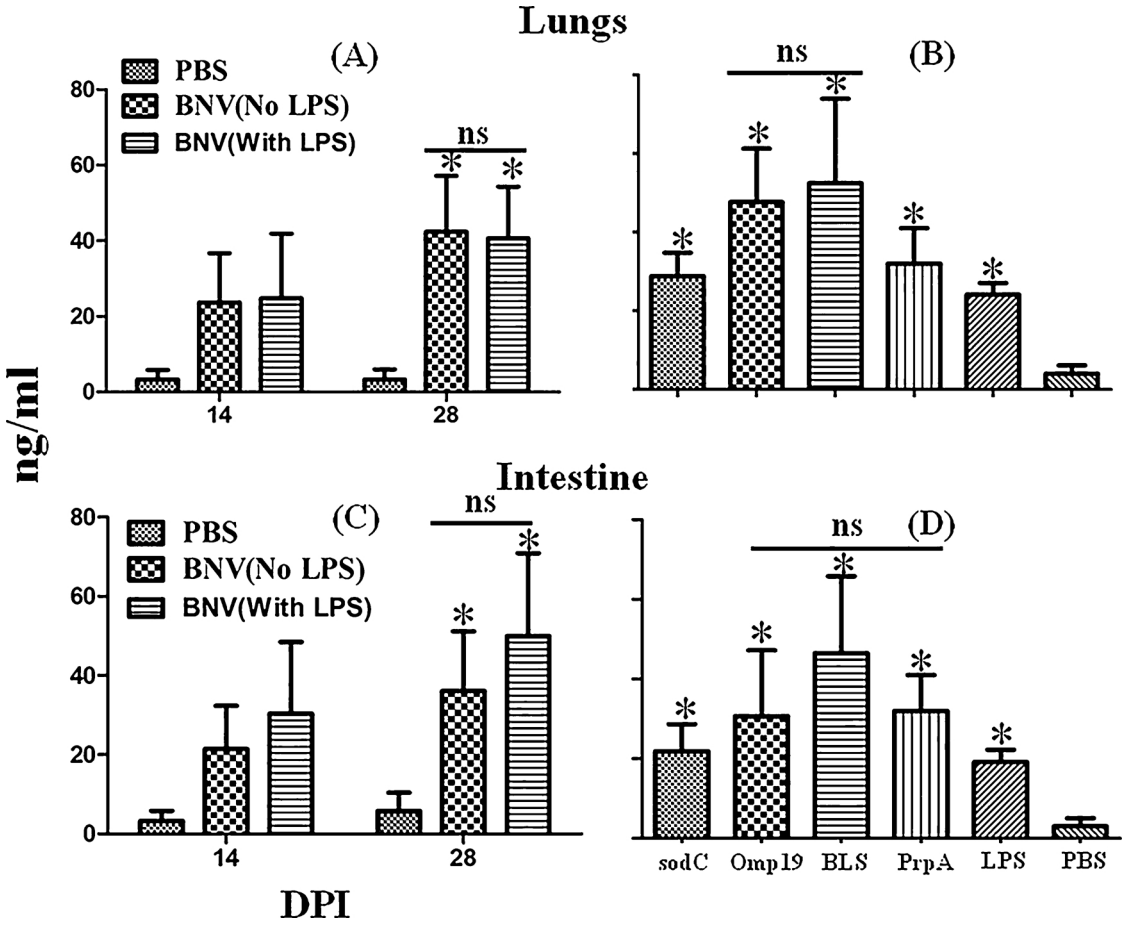

Fig. 2. Mucosal IgA responses.

Mice (n = 16) were intranasally vaccinated with PBS, BNV or BNV plus LPS formulation and intestinal and lung wash samples were collected at 14 (n = 4) and 28 (n = 4) days post-primary immunization for IgA analysis by an indirect ELISA using *Brucella* whole cell lysate (A & C). IgA responses against each individual component of the BNV plus LPS formulation were performed on lungs and intestines samples collected from BNV plus LPS treated mice at 28 days post-primary immunization (B & D). Each data points represent mean ± SD of four animals. * Indicates significant difference compared to PBS control; $p < 0.05$, ns; non-significant, DPI; days post-immunization.

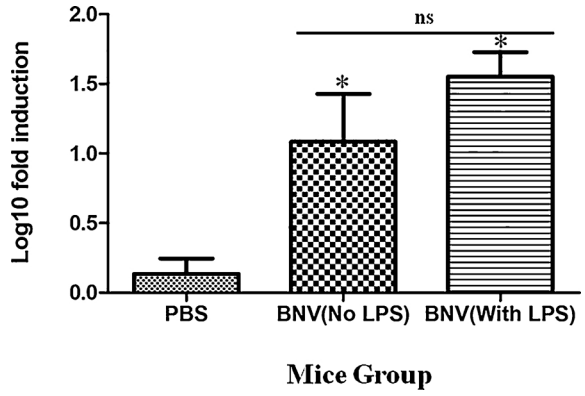

Fig. 3. IFN-γ responses by qRT-PCR assay.
Splenocytes were harvested from vaccinated and control mice (n = 4) on the 14th-day post-primary immunization. Splenocytes (1×10^6 cells/ml) were stimulated with *Brucella* whole cell lysate (10 μg/ml) for 24 h and total RNA was isolated. Subsequently, IFN-γ induction was assayed at mRNA transcript level using qRT-PCR. Gene expression was normalized against beta actin endogenous control. Naïve PBS control was used for calibration purpose. Each data point indicates mean log10 fold induction ± SD of four animals. * Indicates significant difference compared to PBS control; $p < 0.05$. ns; non-significant.

3.4. Protection efficacy against nasal Brucella challenge

To investigate the protective efficacy of BNV vaccine formulation, vaccinated and control mice (n = 8) were intranasally challenged 30 days post-primary vaccination with a virulent wild-type *B. abortus*544 strain. Mice were euthanized on the 14th-day post-challenge and the *Brucella* bacterial load in spleen and lungs were evaluated. Our results showed that mice vaccinated with either BNV plus LPS or BNV alone had significantly ($p < 0.05$) lower bacterial load in both lungs (Fig. 6A) and spleen (Fig. 6B) compared to the PBS control group. Compared to PBS control group, mice that received BNV plus LPS formulation showed 13.1 folds bacterial load reduction in spleen and 2.6 folds reduction in lungs, while as BNV alone formation resulted in 5.4 and 5.8 folds reduction in spleen and lungs, respectively. We further

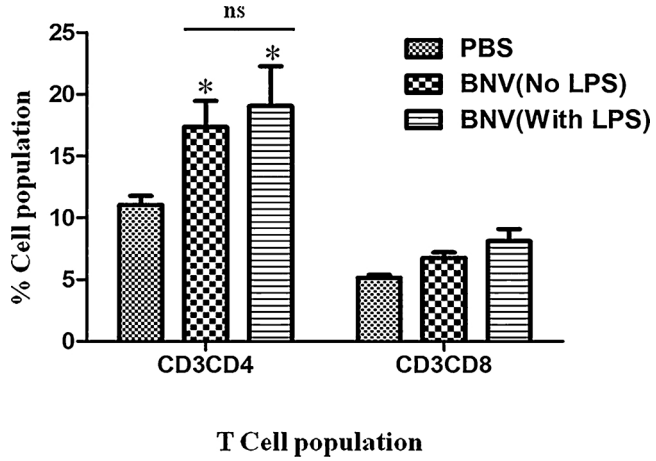

Fig. 5. Flow cytometric analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ splenic T cell population.
The changes in $CD3^+CD4^+$ and $CD3^+CD8^+$ cell subpopulations of the PBS control and the immunized mice groups were assessed in flow cytometry (n = 4). The subpopulations are presented as a percentage of $CD4^+$ and $CD8^+$ T cells subsets gated from $CD3^+$ population. The data was expressed as mean percentage ± SD of four animals. * Indicates significant difference compared to the PBS control group; $p < 0.05$. ns; non-significant.

conducted histopathological analysis of infected lungs and spleens isolated from challenged mice. Our results showed that *Brucella* challenge did not cause any profound lesions in lungs and spleen of vaccinated groups, while as signs of exudate fillings in lung spaces were observed in non-immunized challenged mice group (Fig. 7).

4. Discussion

Development of a safe and effective mucosal vaccine for *Brucellosis* is particularly important since *Brucella* species can effectively enter via mucosal surfaces (Poester et al., 2013) It is also known that *Brucella* species are highly infectious via aerosols in the respiratory route

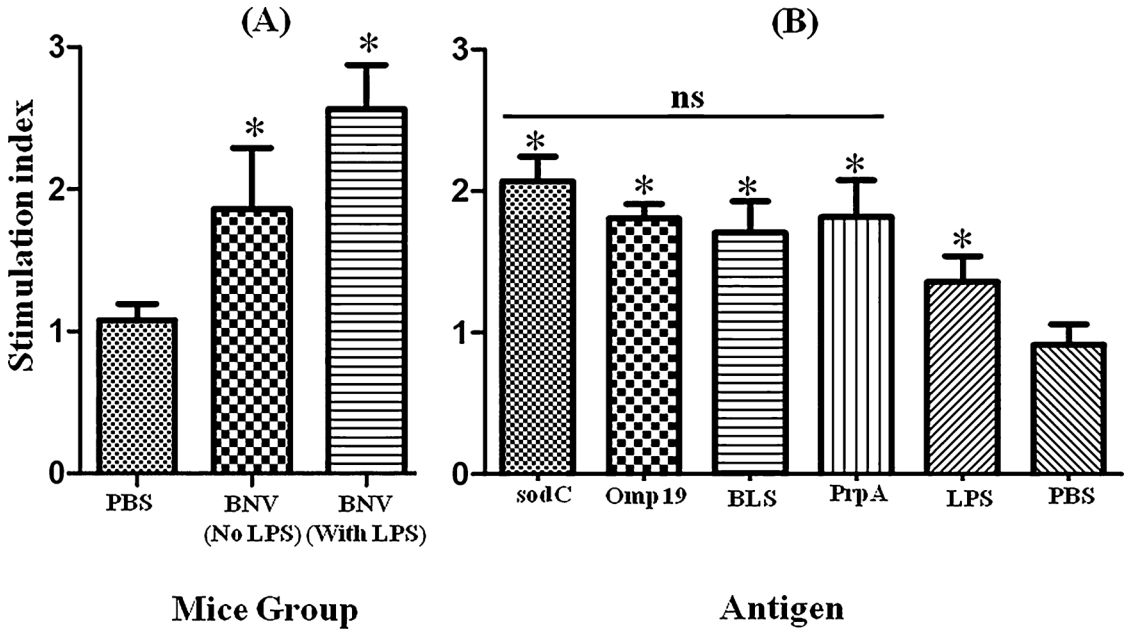

Fig. 4. Lymphocyte proliferative responses.
Splenocytes were harvested from vaccinated and control mice (n = 4) on day 14 post-primary immunization and re-stimulated with either *Brucella* whole cell lysate (A) or individual component of the BNV plus LPS formulation (B) using splenocytes collected from BNV plus LPS treated mice. Each data point indicates mean stimulation index ± SD of four animals. * Indicates significant difference compared to PBS control group; $p < 0.05$. ns; non-significant.

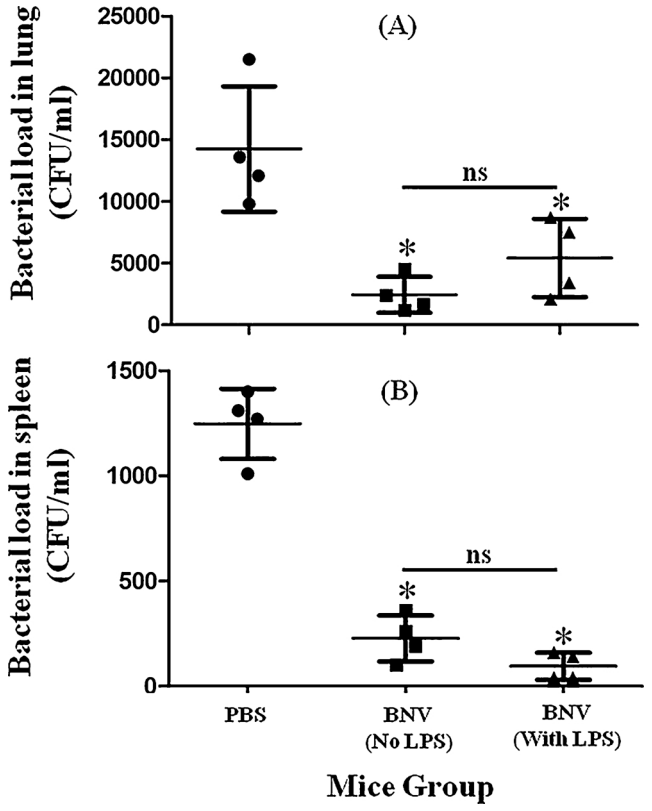

Fig. 6. Protective efficacy of BNV formulation.
Mice (n = 8) from intranasally vaccinated with PBS, BNV or BNV plus LPS formulation were challenged with sub-lethal dose (2×10^4 CFU/mice) of virulent *B. abortus* 544 at 30 days post-primary immunization. Fourteen days after the challenge, four mice were euthanized and challenged *Brucella* was recovered from the lungs (A) and spleen (B). The data was expressed as the mean CFU/ml ± SD of four mice per group. * Indicates significant difference compared to the PBS control group; $p < 0.05$. ns; non-significant.

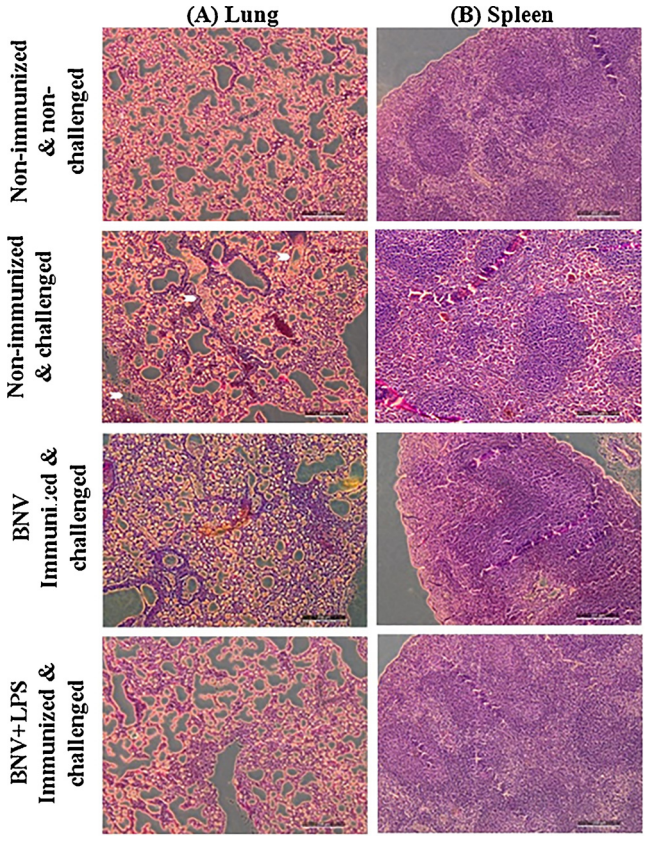

Fig. 7. Histopathological analysis of lungs and spleen isolated from challenged mice.
Mice (n = 8) from intranasally vaccinated with PBS, BNV or BNV plus LPS formulation were challenged with sub-lethal dose (2×10^4 CFU/mice) of virulent *B. abortus* 544 at 30 days post-primary immunization. Fourteen days after the challenge, four mice were euthanized and lungs (A) and spleen (B) were aseptically isolated for histopathological analysis. Paraffin fixed tissue sections were subjected to eosin and hematoxylin staining and observed under the bright filed. Scale bar stands for 200 µm. White arrows indicate exudates filled spaces in lungs of non-immunized mice.

(Doganay and Doganay, 2013) Therefore, a vaccine that can provide protection in the respiratory route by activating adaptive immunity would be highly advantageous. Contemplating this idea, we have formulated BNV formulation and in the current study, we intended to investigate whether newly prepared BNV formulation can elicit *Brucella*-specific systemic and mucosal immune responses and subsequent protection against the sub-lethal *Brucella* nasal challenge. Previous studies have reported that site-specific immunization is advantageous in eliciting mucosal immunity (Clapp et al., 2016), and, therefore, to gain the maximum mucosal immunity in the nasopharyngeal region, we have chosen the intra-nasal route as the preferred route of administration of vaccine antigens. By this procedure, we attempt control *Brucella* at the site of acquisition (mucosal tissues in the nasal region) which may prevent or reduce systemic entry. It was also reported that, parenteral administration of vaccination provides least protection against the pulmonary *Brucella* challenge in mice model (Clapp et al., 2016). In accordance with this notion, we intra-nasally administered our BNV formulation in mice model and evaluated the efficacy of the vaccine via sub-lethal *Brucella* nasal challenge. Instead of high challenge dose usually employed in vaccine trials, the sub-lethal dose will enable us to investigate the efficacy against accidental exposure levels of *Brucella* infection in the natural environment. Here, we demonstrate that vaccination with BNV formulation with or without LPS induced efficient humoral and cell-mediated immune responses and significant protection against the nasal challenge.

In the present study, vaccination of mice with BNV formulation with or without *Brucella* LPS has efficiently elicited both systemic IgG and mucosal IgA response. *Brucella*-specific antibody responses can be particularly important in early phase of *Brucella* infection when the opsonization and complement killing perform a key role in preventing the pathogen from establishment into an intracellular infection (Vitry et al., 2014; Lalsiamthara and Lee, 2017). Consequently, upon intranasal challenge with the virulent *Brucella* abortus 544 strain, significant reduction of *Brucella* infection was observed in vaccinated mice compared to the PBS control group. The present study showed that co-administration of *Brucella* antigens efficiently elicited both IgG and IgA responses against each individual protein component of the BNV formulation. Interestingly, *Brucella* LPS engaged in relatively low induction of IgG and IgA responses and did not make a significant contribution in enhancing the efficacy of the vaccine formulation as anticipated. The lack of immunogenicity of *B. abortus* 544 LPS could be due to non-canonical lipid A structure and altered pathogen-associated molecular patterns of *Brucella* LPS which may interfere with the innate immune response. Also, the concentration of LPS (5 µg/mice) used in the BNV formulation may be insufficient to induce a significant response via intra-nasal route of administration as some reports have shown the requirement of high concentration of *B. abortus* LPS in order to activate TLR4 receptors (Barquero-Calvo et al., 2007). The cell-mediated immunity is considered as a crucial factor in determining the effective clearance of intracellular pathogens such as *Brucella* species (Yingst and Hoover, 2003). IFN-γ, a Th1 type cytokine, is critical in providing protection against intracellular *Brucella* infection and clearance from the infected host (Clapp et al., 2011, 2016; Lalsiamthara and

Lee, 2017). The present study demonstrated that intranasal administration of BNV formulation has induced higher levels of IFN-γ responses in vaccinated mice and this finding was further corroborated with the results of lymphocyte proliferation and $CD4^+$ and $CD8^+$ T cell responses. These results might explain why BNV formulation had significantly reduced bacterial load in both lungs and spleen. The reduction in bacterial load observed in the vaccinated mice was correlating with the results of histopathological analysis performed on lungs and spleen recovered from challenged mice. No significant pathological consequences were observed in any of the spleen or lung tissue sections of vaccinating mice, while as signs of exudates filling in lung tissues of PBS control group was observed. The findings of present study clearly demonstrate that our BNV formulation has potential to offer significant protection against the respiratory exposure of *Brucella* infection.

In summary, we show that intranasal administration of BNV formulation constituted of *Brucella* protective antigens, namely sodC, omp19, BLS and PrpA can induce *Brucella*-specific humoral and cellular immune responses and confer significant protection against sub-lethal nasal *Brucella* challenge. Further improvements related to BNV formulation may be desirable to enhance its productivity, such as dose optimization and/or decreasing the number of constituents to three or two only. The importance of the current study may be justified, since there is no mucosal subunit vaccine approved, so far, to control human brucellosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JHL, AS and JL conceived and designed the study. AS, JL and CH carried out the experiment. AS, IH and JHL wrote and edited the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.11.022.

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