Induction of Th2-related immune responses and production of systemic IgA in mice intranasally immunized with *Brucella abortus* malate dehydrogenase loaded chitosan nanoparticles

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ABSTRACT

The aim of this study was to investigate the induction of mucosal immune responses by an important *Brucella abortus* antigen, malate dehydrogenase (Mdh), loaded in mucoadhesive chitosan nanoparticles (CNs) and immunized intranasally in a BALB/c mouse model. The production of cytokines was investigated in human leukemic monocyte cells (THP-1 cells) after stimulation with the nanoparticles. Mdh-loaded CNs (CNs-Mdh) induced higher interleukin (IL)-6 production than unloaded antigens and TF loaded CNs (CNs-TF). Using ELISpot to quantify cytokines and antibody-secreting cells in the intranasally immunized mice, IL-4 and IgG-secreting cells were found to be significantly increased at 4 weeks and 6 weeks post-immunization in the CNs-Mdh immunized group, respectively. Increases in Mdh-specific IgG, IgG1, and IgG2a antibodies were confirmed at 6 weeks after immunization, indicating a predominant IgG1 response. Analysis of the mucosal immune response in the intranasally immunized mice revealed, Mdh-specific IgA and total IgA in the nasal washes, genital secretions, fecal extracts and sera that were remarkably increased in the CNs-Mdh-immunized group compared to the CNs-TF-immunized group except total IgA of nasal wash. Therefore, the results indicated that the intranasal immunization of CNs-loaded *B. abortus* Mdh antigen effectively induced antigen-specific mucosal immune responses through the elicitation of Th2-related immune responses.

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1. Introduction

Brucellosis is a globally widespread zoonotic disease that is transmitted from domestic animals to humans, either directly or indirectly through contact with infected animals or animal products. The occurrence of the human brucellosis depends largely on the occurrence of brucellosis in an animal reservoir. *Brucella* is classified into eleven species according to host specificity, and four species infect humans [1,2]. Of these, *B. melitensis* and *B. abortus* are the main causative agent of brucellosis in both ruminants and humans. *B. abortus* has also wide spread in wild-life including feral pigs, buffalo, bison and elk. For example, *B. abortus* wild-type and the *B. abortus* S19 and RB51 vaccine strains have isolated from feral pigs in areas that have not raised any livestock since 1970 [3]. *B. abortus* is a causative organism of bovine brucellosis and can be a major threat to human health due to its high infectivity. The transmission of brucellosis to humans occurs through a variety of routes including the ingestion of unpasteurized animal-dairy products, direct contact with infected animal parts, and inhalation of infected aerosolized particles [4]. In this regard, focusing on stopping the spread of disease through the use of effective and safe vaccines to prevent and control both human and bovine brucellosis is important.

The live attenuated *B. melitensis* vaccine strain Rev.1 is recognized worldwide as a vaccine available against sheep and goat brucellosis. To prevent brucellosis in small ruminants, the conjunctival immunization is considered a better route of administration. This

is because vaccination of young animals using the conjunctiva route instead of the subcutaneous route significantly shortens the serological response [5]. And conjunctiva infected *B. melitenses* can be induced IgG+ cells and IFN-γ producing T cells from mucosal lymph node which leads to the control of infection [6]. Strains 19 and RB51 are the *B. abortus* vaccine strains most commonly used to protect cattle against infection and abortion [7]. However, as these vaccines have several disadvantages, much effort has been devoted to developing a safe and effective subunit vaccine with recombinant proteins [8–12]. Malate dehydrogenase (Mdh), a key enzyme in the tricarboxylic acid (TCA) cycle (Fig. 1). Mdh elicited high cytokine production and toll-like receptor (TLR) expression in human leukemic monocyte cells (THP-1) cells that were stimulated by several recombinant proteins [13]. In addition, vaccination via the intraperitoneal injection of purified *B. abortus* Mdh was reported to significantly reduce colonization and cause the rapid elimination of S19 in BALB/c mice [14]. However, traditional vaccination strategies, which require multiple injections using needles or other invasive routes, have problems with administration, needle cross-contamination, cost and patient compliance [15]. In this regard, mucosal immunization using recombinant proteins is a very attractive vaccine choice because of its safety and effective induction of immunity through natural routes without needles.

The mucosal surface serves as the first line of defense against infection as it is the initial contact site for pathogens [16]. The induction of secretory IgA from the mucosa blocks bacterial and viral adhesion to the mucosal surface and prevents damage to the host. The mucosal immune response is most effective when a vaccine is administered to the mucosal surface; however, injection vaccines do not always reach mucosal surfaces and are consequently less effective at preventing mucosal surface infections [17]. Secretory IgA can be induced after being immunized with various mucosal pathways such as oral, nasal and ocular route. In case of the ocular route, invasive procedures are required to induce additional immunity, as it is associated with low productivity and difficult to control dose [18]. However, the intranasal route shows relatively less proteolytic activity which can protect protein antigens from enzymatic degradation, thus requiring fewer antigens than the oral route [19]. Additionally, the nasal epithelium is highly permeable and vascularized with cervical lymph nodes and lymphoid cells [19]. Therefore, antigens are easily accessible if they can be transported properly through the epithelium. However, despite these beneficial features, one of the most important limitations of nasal immunity is the rapid mucociliary clearance that occurs at the mucosal surface, which hinders antigen delivery because the immunogen formulation is rapidly removed from the mucosa. Materials administered through the nasal cavity have been reported to be removed from the nasal membranes within approximately 21 min via mucociliary clearance [20]. Therefore, increasing the residence time of antigens in the nasal cavity to increase their contact time with the nasal mucosa to induce effective nasal mucosal immunity is necessary.

Effective adjuvants are needed for antigen delivery systems because recombinant protein antigens delivered to mucosa are often not highly immunogenic. Recently, chitosan has been attracting attention as a promising polymer due to its nontoxic, biocompatible, and biodegradable nature, as well as its mucoadhesive and permeation-enhancing properties [21,22]. Chitosan is produced from chitin by deacetylation, and chitin is a substance commonly found in natural crustaceans and is known to affect the immune response [23]. Nanoparticles or microspheres are very common forms used in controlled vaccine and drug delivery formulations. Chitosan particles increase the immune response against influenza, diphtheria and pertussis in human vaccines, and are also used as delivery systems for veterinary vaccines for preventing atrophic rhinitis, piglet paratyphoid and porcine pleuropneumoniae. [21,24–26]. A spherical form is known to increase the residence time of vaccines on the mucosal surface compared to solutions because the sphere absorbs water from mucus and dehydrates the epithelial cells to separate the tight junctions [27]. The chitosan nanoparticles (CNs) used in this study are the most widely studied drug delivery system for the controlled release of antibiotics, anti-hypertensive agents, proteins, peptide drugs, and vaccines [28–30]. Previous studies have shown that CNs enhance mucosal absorption and have adjuvant activity in the mucosal immune response through intranasal administration [21,24].

In the present study, CNs were prepared and characterized as an adjuvant for the controlled release of *B. abortus* Mdh. The immuno-stimulating activity of Mdh-loaded CNs (CNs-Mdh) was measured by examining the systemic and local mucosal immune responses that were elicited through intranasal immunization in mice.

2. Materials and methods

2.1. Purification of B. abortus malate dehydrogenase

The bacterial strains were the same as those used in a previous study [13]. Each *E. coli* DH5α clones containing a recombinant
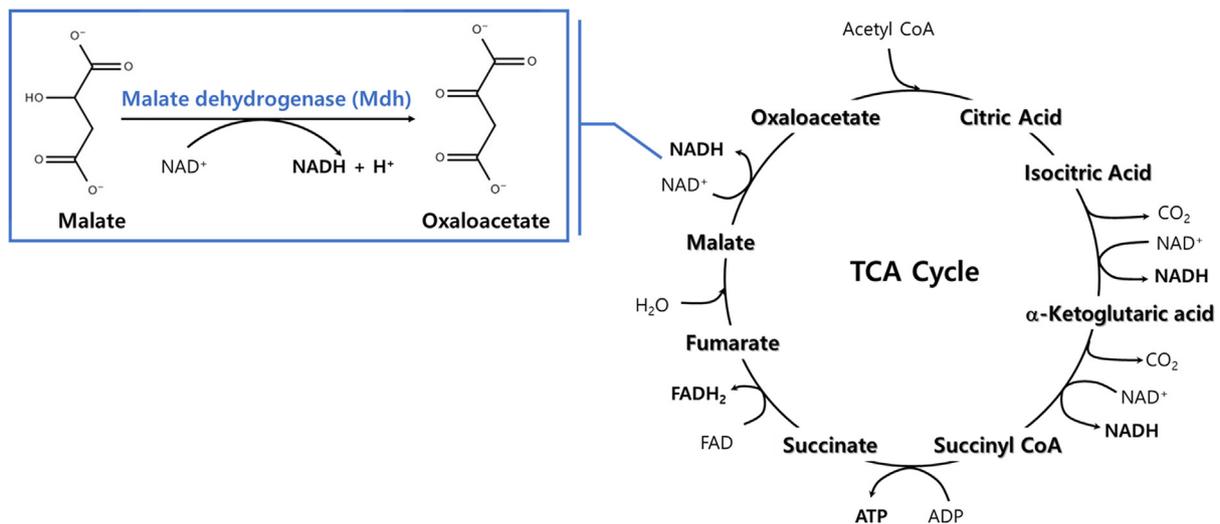

Fig. 1. Tricarboxylic acid (TCA) cycle and the position of Malate dehydrogenase (Mdh). This image was modified from Google's image search.)

plasmid was grown in Luria Bertani (LB) agar supplemented with ampicillin (100 µg/ml). A single colony was inoculated into 60 ml of LB broth (100 µg/ml ampicillin), which was then incubated at 37 °C overnight with agitation at 220 rpm. Sixty milliliters of each culture were inoculated into 1 L of terrific broth containing 100 µg/ml ampicillin and grown under the same conditions to an OD_{600} of 1.5. Cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Amresco, USA), and incubation was continued with shaking at 15 °C for 12 h. The bacterial cell pellets were then harvested and resuspended in binding buffer (20 mM Tris–HCl, 8 M urea, 500 mM NaCl, 20 mM imidazole [pH 8.0], and 1 mM β-mercaptoethanol (Sigma, USA)). Each bacterial suspension was sonicated on ice, and the supernatants were collected after centrifugation at $3000\times g$ for 20 min. The recombinant proteins were purified under denaturing conditions using a His SpinTrap (GE Healthcare, UK) according to the manufacturer's protocol. Protein concentration was determined via Pierce 660 nm protein assays (Thermo Scientific, USA). The recombinant proteins were analyzed by SDS-PAGE and Western blotting with a 6x-His Tag Monoclonal Antibody (Thermo Scientific, USA).

2.2. Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared based on the ionic gelation of chitosan with sodium tripolyphosphate (TPP) anions. Water-soluble chitosan provided by Jakwang (deacetylation degree of 82.4%, Ansung, Korea) was dissolved in distilled water (0.5 wt-%), and 300 µl of an 8% Tween-80 solution was added to 50 ml of the chitosan solution. Using a 26G needle, 500 µl of an aqueous TPP solution (10 wt-%) was added dropwise into 50 ml of the water-soluble chitosan solution (0.5 wt-%) containing Tween-80 under magnetic stirring and sonication (6 W, 10 min) on ice. Chitosan nanoparticles were obtained by centrifugation at 14,000 rpm for 20 min and were freeze-dried after being rinsed with distilled water.

2.3. Loading of recombinant proteins

Twenty milligrams of chitosan nanoparticles were dispersed in 1 ml of protein solution (4 mg/ml, pH 7.4 PBS). The suspension was kept at 37 °C for 24 h with continuous shaking, followed by centrifugation at 8,000 rpm for 10 min to remove unloaded proteins. The loading efficiency was measured by quantifying the unloaded protein in the supernatant via Pierce 660 nm protein assays. The loading efficiency (%) was calculated using following equation.

$$\text{Loading efficiency}(\%) = \frac{\text{Total amount of protein} - \text{unloaded protein}}{\text{Total amount of protein}} \times 100$$

2.4. Characterization of chitosan nanoparticles

The particle size distribution of the CNs was measured by dynamic light scattering (DLS) using a zeta potential and particle size analyzer (ELSZ-1000, Otsuka Electronics Ltd., Japan). The freeze-dried nanoparticles were observed using a field-emission scanning electron microscope (FE-SEM, Supra 55VP, Carl Zeiss, Germany) after being coated with platinum.

2.5. In vitro studies

2.5.1. Release test

Ten milligrams of TF-loaded CNs (CNs-TF) and Mdh-loaded CNs, each suspended in 1 ml of PBS (pH 7.4) in a 1.5-ml tube, were agitated for up to 24 h at 37 °C and 220 rpm using a shaking

incubator. After centrifugation (8000 rpm, 10 min), 1 ml of supernatant was obtained at each time point, and the same amount of fresh PBS was added. The amount of released trigger factor (TF) and Mdh protein was determined as cumulative release (wt.-%) over incubation time by Pierce 660 nm protein assays.

2.5.2. Immunocytochemistry in THP-1 cells

Differentiated THP-1 macrophage cells (5×10^5 cells/well) were stimulated with Mdh alone (10 µg/well) and Mdh-loaded CNs (10 µg/well). Cells were fixed with 4% paraformaldehyde for 10 min at RT and then permeabilized in ice-cold 0.5% Triton X-100 10 min at RT. After three washes with 0.1% PBS-T (0.1% Tween 20 in phosphate buffered saline), the samples were blocked with blocking buffer (1% bovine serum albumin in 0.1% PBS-T) for 1 h at RT, subsequently incubated with a 1:500 dilution of primary antibody (6×-His Tag Monoclonal Antibody, Thermo Scientific) in blocking buffer for overnight at 4 °C. After three washes with 0.1% PBS-T, cells were incubated with a 1:200 dilution of an Alexa 488-conjugated Goat anti-mouse IgG2b (Invitrogen, USA) for 1 h at RT. Cells were washed in 0.1% PBS-T and mounted using Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratory, Burlingame, CA). Images were taken 2 h, 6 h and 12 h after stimulation of Mdh and CNs-Mdh with a confocal microscopy (Zeiss LSM 800, Germany)

2.5.3. Measurement of secreted cytokines in THP-1 cells

Human leukemic monocyte cells, THP-1 cells, were stimulated as previously described [13]. Enzyme-linked immunosorbent assays (ELISA) were performed to evaluate cytokine secretion into the cell culture supernatant. Differentiated THP-1 macrophage cells (1×10^6 cells/well) were stimulated with recombinant proteins (10 µg/well) and protein-loaded CNs (10 µg/well for each protein). LPS (10 µg/well, Sigma, USA) and DPBS were included as positive and negative controls, respectively. The culture supernatants were collected at 6 h, 12 h and 24 h after stimulation. All stimulants were pre-treated with polymyxin B (10 µg/ml) before stimulation of cells. The amounts of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were measured by ELISA according to the manufacturer's instructions (ThermoFisher, USA).

2.6. In vivo studies

2.6.1. Intranasal immunization of mice

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (IACUC approval number: SNU-170504-5-3). The nasal immunization study was conducted with 6-week-old female BALB/c mice, which were divided into four groups (n = 12). The mice in each group were immunized on days 0, 14 and 28 by dropping 20 µl of PBS containing TF (30 µg)-loaded CNs, Mdh (30 µg)-loaded CNs or CNs alone into the nostrils under anesthesia. Sera, feces, and nasal and genital secretion samples were collected from each group every two weeks after the first immunization. Briefly, blood samples were obtained by cardiac puncture and were left to stand at 4 °C overnight. Sera were collected by centrifugation at 1000g for 10 min at 4 °C. Two or three fecal pellets were freshly collected from each mouse. PBS (10 wt-%) with protease inhibitor cocktail powder (Sigma) was added to the fecal samples, which were then vortexed and centrifuged at 16,000g for 5 min at 4 °C. The supernatants were used for ELISAs. Nasal fluid was obtained by flushing the nasal cavity from the pharynx through the nostrils with 300 µl of PBS containing protease inhibitor cocktail. To prepare the genital secretions, the uterine horn through the vaginal region was separated, placed in 1 ml PBS containing protease inhibitor and vortexed. Centrifugation was performed at 16,000g for 10 min at 4 °C, and the supernatants were

collected. All of the prepared samples were stored at $-70\,°C$ for further analysis.

2.6.2. Elispot assays for total IgG, IFN-γ and IL-4

Total IgG-producing B cells and IFN-γ and IL-4-secreting T cells from the spleens of 4 and 6 weeks post primary immunization (wpi) mice were measured using an Enzyme-Linked ImmunoSpot (ELISpotBasic) assay kit according to the manufacturer's instructions (Mabtech AB, Sweden). For total IgG-producing B cells, the ELISpot plates were pretreated with 70% ethanol and then to coat the plates, 100 µl of antibody solution (anti-IgG antibody;15 µg/ml) was added and incubated overnight at 4 °C. For IFN-γ and IL-4-secreting T cells, 200 µl of monoclonal antibodies against IFN-γ (AN18, 15 µg/ml) and IL-4 (11B11, 15 µg/ml) were added to the plates and coated as described above. After incubation, the plates were washed with PBS five times and blocked with RPMI1640 containing 10% FBS for 30 min at room temperature (RT). After removal of the medium, splenocytes isolated from mice at 4 and 6 wpi were added at a concentration of $1–2.5 × 10^5$ cells/well. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h for IgG or 48 h for IFN-γ and IL-4. After removing the cells, 100 µl of biotinylated anti-IgG, IL-4, or 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 was added and incubated for 1 h at RT. Antigen-secreting cells (ASCs) were visualized upon the addition of ready-to-use TMB substrate solution after washing the wells with PBS. The numbers of ASCs were counted using Eli. Scan+ (A.EL.VIS, Germany). These experiments were repeated three times.

2.6.3. Measurement of antibody production

Specific indirect ELISAs were performed to determine the IgG, IgG1, IgG2a and IgA titers. Briefly, using purified recombinant Mdh, each well of flat-bottom 96-well plates (Greiner Bio-One, Monroe, NC) was coated with 10 ng/well of bicarbonate buffer (pH 9.6), followed by incubation at 4 °C overnight. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and were blocked at RT with 3% (w/v) BSA in PBS-T to prevent non-specific binding. For neutralizing TF-specific antibodies, samples were pre-incubated with purified TF protein (20 µg/well) at 37 °C for 1 h. The plates were incubated with appropriately diluted samples at RT for 2 h. The sera samples were diluted 1:1000 to detect specific IgG, IgG1 and IgG2a. For specific IgA measurement, the nasal wash samples were diluted 1:80, the genital secretions were diluted 1: 800, the fecal extracts were diluted 1:25 and the sera were diluted 1:8000. Goat anti-mouse IgG (Bio-Rad Laboratories, USA), IgG1 (Southern Biotechnology, USA), and IgG2a (Southern Biotechnology, USA) and Rabbit anti-mouse IgA (BioFX Laboratories, USA) HRP conjugates diluted 1:2000 (100 µl/well) were added to the wells and incubated at RT for 1 h. Following the addition of 100 µl of TMB as a substrate, the plates were reacted at RT for 10 min. Finally, color development was stopped by the addition of 100 µl of 1 N H_2SO_4, and the optical density of each well was measured at 450 nm using a VersaMax microplate reader (Molecular Devices Corporation, CA, USA). Total IgA in the nasal wash, genital secretion, fecal extract and serum samples was measured using a Mouse IgA ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.7. Statistical analysis

All the data given as the mean ± SD were analyzed by Welch's t-tests and one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparison tests using Graphpad Prism version 5

(San Diego, CA). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 were considered statistically significant.

3. Results

3.1. SDS-PAGE and Western blotting

The pCold TF DNA vector is a fusion cold shock expression vector that expresses TF, an approximately 48 kDa ribosome-associated chaperone protein, as a soluble tag. The TF and Mdh proteins were characterized by SDS-PAGE and Western blot analysis. As shown in Supplementary Fig. 1, the SDS-PAGE profiles indicated that purified TF and Mdh were 52 kDa and 85.71 kDa in size, respectively. The recombinant proteins, which were expressed from the pCold TF vector and purified using a histidine column, were confirmed by Western blotting with an anti-histidine antibody.

3.2. Characterization of recombinant protein-loaded CNs

Nanoparticle morphology was observed via scanning electron microscopy (SEM). SEM photographs showed that the CNs were spherical with a smooth-surface and uniform size (Fig. 2A). The CNs-TF and CNs-Mdh, however, were aggregated and large with bumpy surfaces compared to the CNs. The sizes of the CNs, CNs-TF and CNs-Mdh, as measured by DLS, were 324.6 ± 90.1 nm, 1279.0 ± 116.8 nm and 664.6 ± 161.7 nm, respectively (Fig. 2B). As seen from the DLS results, the size distributions of all particles were uniform with a single peak.

3.3. In vitro studies

#### 3.3.1. Loading efficacy measurements and release tests for the protein-loaded CNs

The loading efficiencies of the recombinant proteins in the CNs are shown in Supplementary Table 1. The loading efficiencies of TF and Mdh were 61.23 ± 3.58% and 50.97 ± 0.82% respectively (n = 6). TF showed similar loading efficiency in each tube and was approximately 10% higher than that of Mdh. Fig. 3 shows the percentage of recombinant protein released from the nanoparticles. The profile showed that 27% and 39% of TF and Mdh was released from the CNs at up to 80 h, respectively. TF and Mdh were released from the nanoparticles through a burst release within the first 24 h, which was followed by slow and steady release for next 80 h that reached a plateau.

3.3.2. Immunocytochemistry in THP-1 cells

Confocal microscopy images were taken at 2, 6, and 12 h after stimulation with Mdh alone and CNs-Mdh in THP-1 cells (Fig. 4). The detection of the his-tagged Mdh protein was performed with anti-his antibodies and analysis via confocal fluorescence microscopy. All the images showed that the Mdh protein was localized predominantly in the nucleus in the majority of cells. In the cells stimulated with Mdh alone, the proteins were distributed in the cytosol in the first 2 h, and the cytosolic protein intensity was faint than that in 12 h as time passed. Similarly, when stimulated with CNs-Mdh, the Mdh protein intensity of the cytosol dimmed with time, but Mdh protein was localized predominantly in the nucleus.

3.3.3. Cytokine measurement in stimulated THP-1 cells

Culture supernatant from THP-1 cells was assayed by ELISAs for TNF-α and IL-6 after being stimulated with recombinant proteins and protein-loaded CNs (Fig. 5). The production of TNF-α decreased constantly in a time-dependent manner after antigen stimulation until 24 h. The production of IL-6 increased in a time-dependent manner after antigen stimulation until 24 h. Overall, the
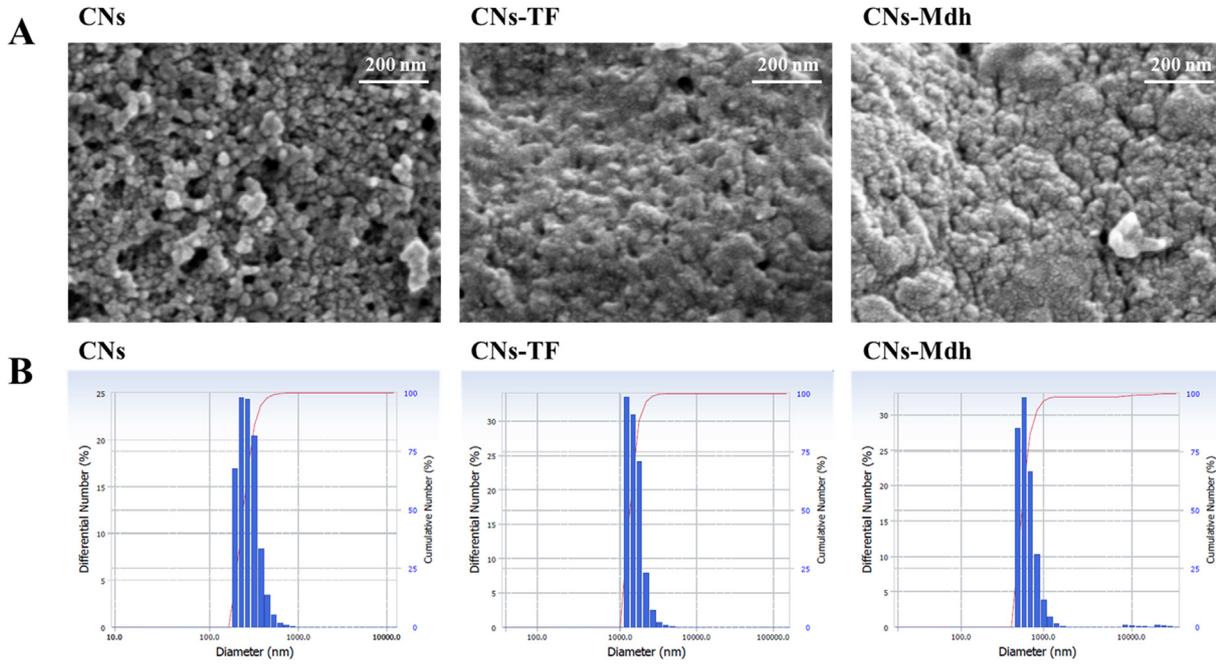
Fig. 2. (A) SEM photographs of the CNs, CNs-TF and CNs-Mdh and (B) the average particle size distribution of the CNs, CNs-TF and CNs-Mdh using dynamic light scattering (ELSZ-1000). *Footnotes:* SEM, Scanning electron microscopy; CNs, Chitosan nanoparticles; CNs-TF, Trigger factor loaded chitosan nanoparticles; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.
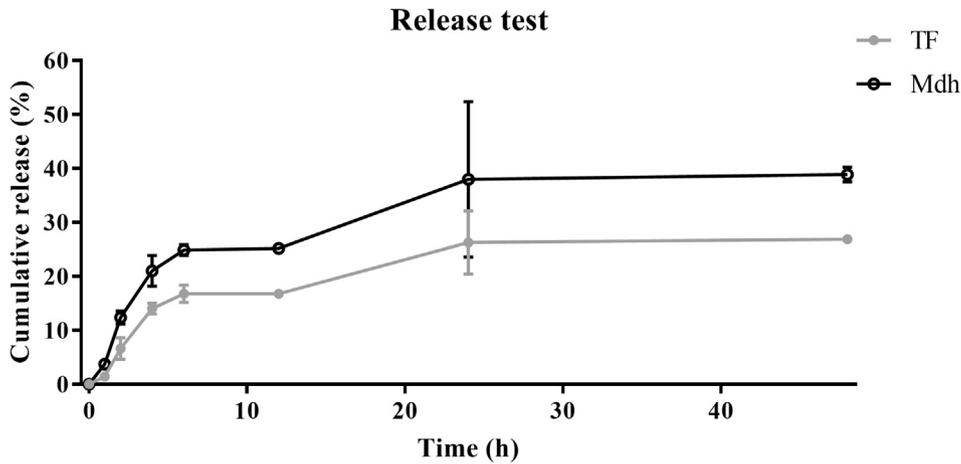

Fig. 3. Release of TF and Mdh from loaded chitosan nanoparticles. *Footnotes:* TF, Trigger factor; Mdh, Malate dehydrogenase.

protein-loaded CNs-treated cells showed lower levels of TNF-α than the protein-treated cells. In the cells treated with protein alone, Mdh elicited significantly higher cytokine levels at 6 h, 12 h, and 24 h when TF and Mdh were compared; however, in the protein-loaded CNs-treated cells, the CNs-Mdh-treated cells had slightly decreased cytokine levels at 12 and 24 h compared to those of the CNs-TF-treated cells. When the TF- and Mdh-treated cells were compared, the Mdh-treated cells showed higher IL-6 secretion at all time points. The IL-6 levels were higher in the protein-treated cells than in the protein-loaded CNs-treated cells at 6 h after stimulation. However, the differences in the IL-6 levels between the protein and protein- loaded CNs-treated cells were reduced at 12 h, and the secretion of IL-6 showed a different trend at 24 h post-stimulation. The IL-6 levels in the protein-loaded CNs-treated cells exceeded those of the protein-treated cells at 24 h. In addition, the highest level of IL-6 was observed in the CNs-Mdh-treated cells, and it was a statistically significant difference when compared with that of the CNs-TF-treated cells.

3.4. In vivo studies

3.4.1. Elispot

To quantify the antibody and cytokine secretory cell numbers, enzyme-linked immunosorbent (ELISpot) analysis was performed using immunized mouse splenocytes. Fig. 6 shows the average numbers of IgG, IL-4 and IFN-γ secreting cells in splenocytes at 4 and 6 wpi. At 4 wpi, the CNs-Mdh-immunized group had significantly increased numbers of IL-4 secreting cells compared to those of the PBS and CNs-immunized groups; however, there were no significant differences among the groups for of IgG and IFN-γ secreting cells. Interestingly, a different trend was observed at 6 wpi. The number of IgG-secreting B cells was significantly increased in the CNs-TF and CNs-Mdh-immunized mice compared to those of PBS and CNs-immunized mice. In addition, when the CNs-Mdh and CNs-TF-immunized groups were compared, the number of IgG-secreting cells was confirmed to be significantly increased in the CNs-Mdh-immunized mice. Unlike 4 wpi, the
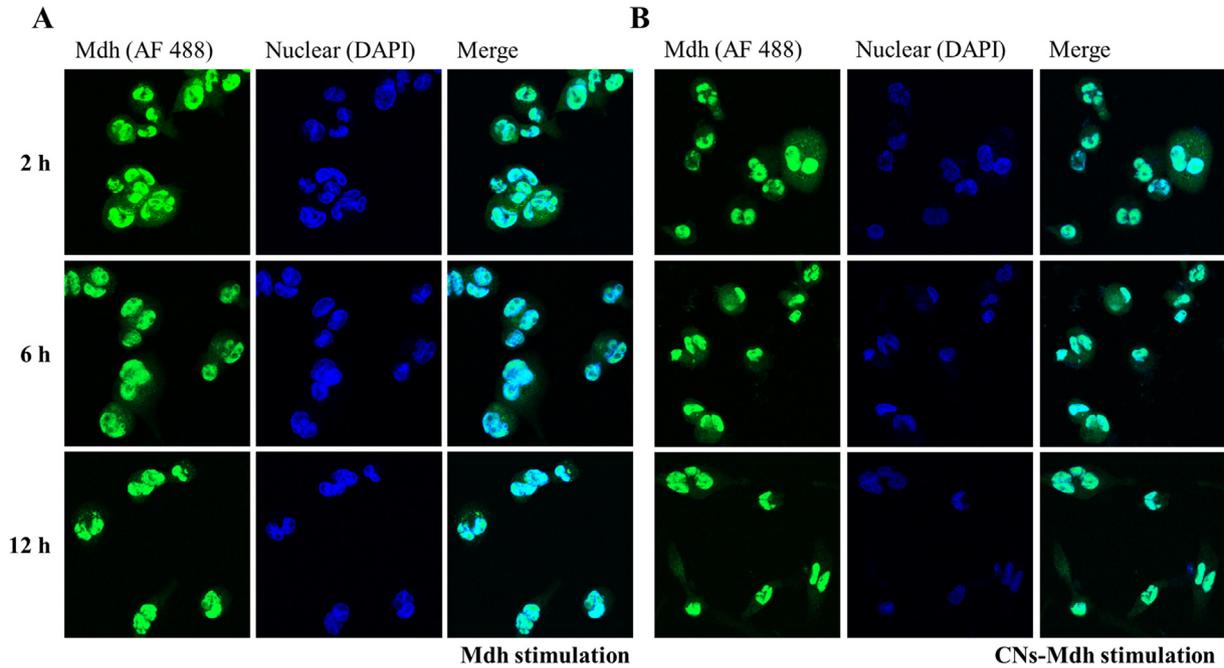
Fig. 4. Confocal microscopic analysis after immunofluorescence staining showing the nuclear localization of anti-his tag antibody conjugated with Alexa Fluor 488 (green) and nuclear staining with DAPI (blue). (A) Confocal images taken at 2 h, 6 h, and 12 h after stimulation with Mdh alone in THP-1 cells. (B) Confocal images taken at 2 h, 6 h, and 12 h after stimulation with CNs-Mdh in THP-1 cells. *Footnotes:* AF, Alexa fluor; DAPI, 4′,6-diamidino-2-phenylindole; Mdh, Malate dehydrogenase; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.
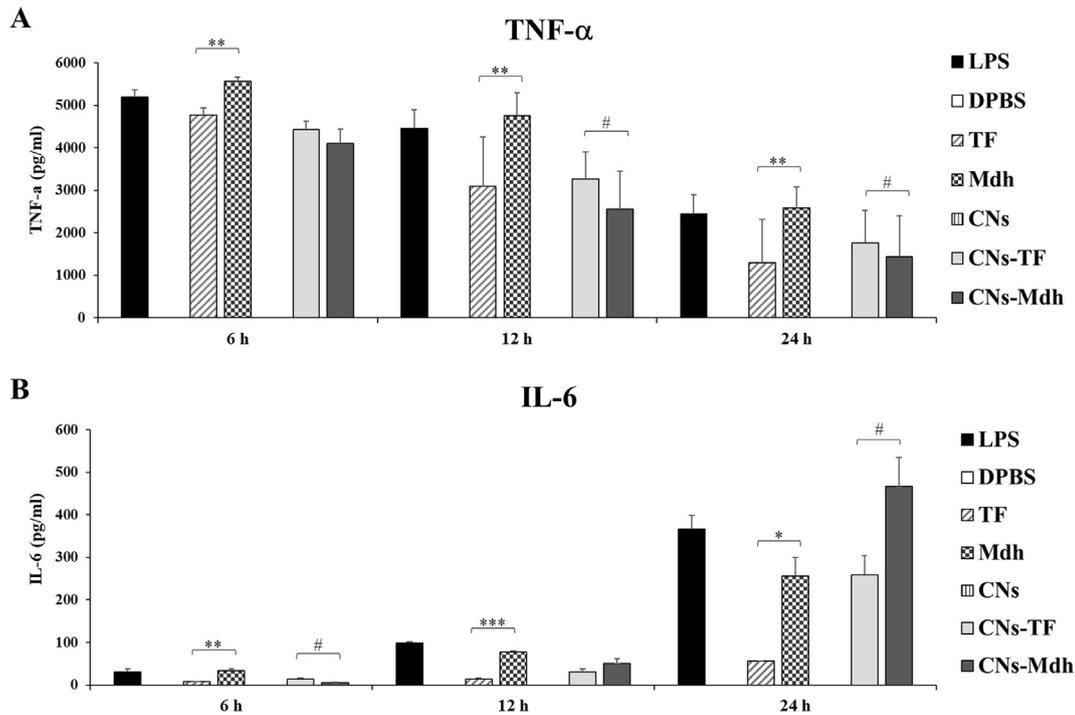

Fig. 5. TNF-α (A) and IL-6 (B) concentrations in the supernatants of THP-1 cells at 6 h, 12 h and 24 h after primary stimulation. Cells (1×10^6) were stimulated with 10 μg of each antigen(■: LPS, □: DPBS, ▨: TF, ⊠: Mdh, ▥: CNs, ▢: CNs-TF, and ▦: CNs-Mdh). Data are expressed the mean ± SD, and statistical analysis was performed using Welch's t-test. *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences between the protein-treated cells (TF and Mdh). #P < 0.05 indicates significant differences between the protein-loaded CNs treated cells (CNs-TF and CNs-Mdh). *Footnotes:* TF, Trigger factor; Mdh, Malate dehydrogenase; CNs, Chitosan nanoparticles; CNs-TF, Trigger factor loaded chitosan nanoparticles; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.

numbers of IL-4-secreting cells were similar in all of the groups. The number of IFN-γ-secreting cells was significantly increased in only the CNs-Mdh-immunized group compared with the PBS-immunized group.

3.4.2. Measurement of Mdh specific antibodies

To study the humoral immune responses in mice induced by nasal immunization, at 6wpi, we measured the IgG, IgG1 and IgG2 levels in sera as well as IgA production in nasal washes,

A

B
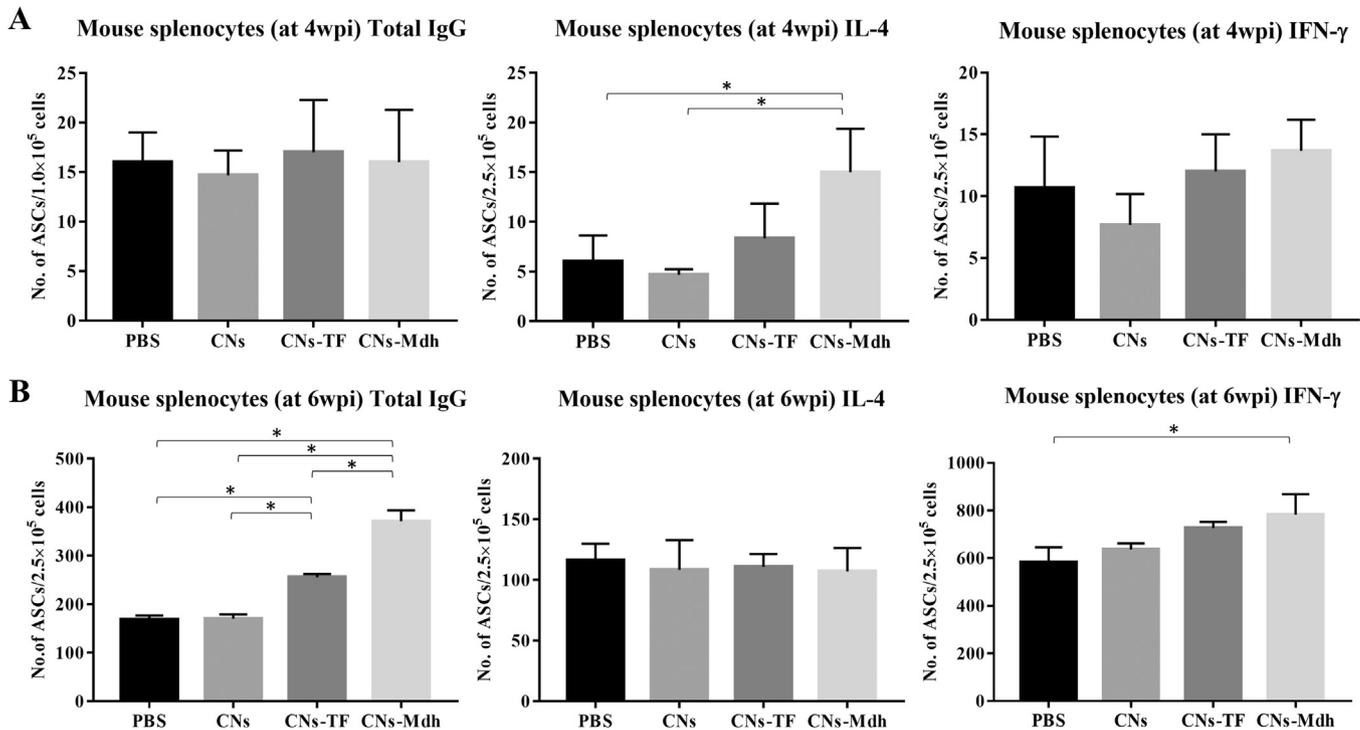
Fig. 6. ELISpot analysis in immunized mouse splenocytes. (A) The numbers of IgG, IL-4 and IFN-γ-secreting cells are shown at 4 weeks after primary immunization. (B) The numbers of IgG, IL-4 and IFN-γ-secreting cells per 2.5×10^5 cells are shown at 6 weeks after primary immunization. Values represent the mean ± SD of three measurements per pool of splenocytes. Groups were statistically compared using one-way ANOVA with Tukey's post hoc multiple comparison test (*$P < 0.05$). *Footnotes:* CNs, Chitosan nanoparticles; CNs-TF, Trigger factor loaded chitosan nanoparticles; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.

genital secretions, fecal extracts and sera (Fig. 7). To determine Mdh-specific antibodies, the TF-specific antibodies were neutralized, measured by ELISAs and used to determine S/N ratios (sample to negative control ratio). A slight but non-statistically significant increase in the serum IgG, IgG1 and IgG2a ELISA results, was observed for the CNs-Mdh-immunized group compared to those of the CNs-TF-immunized group. Overall, the isotype patterns of the two showed a dominance of IgG1 antibodies over IgG2a antibodies. However, Mdh-specific IgA production was elicited significantly more in serum ($P < 0.05$) and all mucosa-related samples, including nasal wash ($P < 0.001$), genital secretion ($P < 0.01$) and fecal extract ($P < 0.05$) samples, compared to the other groups.

3.4.3. Measurement of total IgA

Total IgA levels were measured for the sera, nasal washes, genital secretions and fecal extracts sampled at each period, as shown in Fig. 8 (at 2, 4 and 6 wpi). The serum IgA levels between the CNs-TF and CNs-Mdh-immunized groups were significantly different at all time points. No significant differences were observed in the nasal wash IgA levels between the CNs-TF and CNs-Mdh-immunized groups. For the genital secretions, however, a significant difference was found between the CNs-TF and CNs-Mdh-immunized groups at 2 and 6 wpi, and a very strong IgA increase in CNs-Mdh-immunized group was observed at 6wpi compared to the other groups ($P < 0.001$). Significant IgA responses were detected in the fecal extracts from the groups, with those of the CNs-Mdh-immunized group being significantly higher at 4 and 6 wpi.

4. Discussion

Brucella spp. infects other domestic and wild animal species that can act as a reservoir of disease for other animal species and humans, as well as for their preferred host – goats and sheep (*B.*

melitensis), cows (*B. abortus*) and sows (*B. suis*) [31]. Despite efforts to eradicate disease, all studies show that the prevalence of brucellosis in a variety of countries, such as the Mediterranean, the Middle East and India, is widespread in human [32]. As a mucosal pathogen, *B. abortus* infects humans and animals mainly through the digestive or respiratory tract. Therefore, immune induction against brucellosis at the mucosal surface is required as the first line of defense. To induce mucosal immunity effectively and safely, candidate proteins were selected, and our group previously showed that *B. abortus* Mdh is one of the most promising candidates with effective immunostimulatory activity in THP-1 cells [13]. Mdh is a key enzyme in the TCA cycle, an important process in energy metabolism. For some intracellular organisms such as *Brucella* spp., *Toxoplasma gondii* and *Mycobacterium avium* subsp. *paratuberculosis,* partial protection and immunostimulatory effects elicited by Mdh were shown [33,34]. Additionally, to improve mucosal immunity with an effective antigen protein, establishing an appropriate antigen delivery system for inoculation of the nasal cavity is important. For this reason, we investigated the immunological response elicited by *B. abortus* Mdh, a candidate recombinant protein, by nasal administration using CNs as an adjuvant to induce effective mucosal immunity.

In SEM photographs and DLS results, the protein-loaded nanoparticles had rougher surfaces and were approximately 2–6 times larger in size compared to the CNs alone. Since the SEM photographs were measured in a dry form, differences in size were observed with DLS, where the samples were dispersed in distilled water; the swollen form is larger than the dry form [35]. Taken together, these findings suggest that the proteins were well entrapped on the surfaces of the nanoparticles. When administered intranasally to deliver antigen, proper particle size is critical for absorption into mucosa-associated lymphoid tissue (MALT), as many studies have demonstrated the relationship between particle size and mucociliary clearance in the airways [36,37]. Nano- or
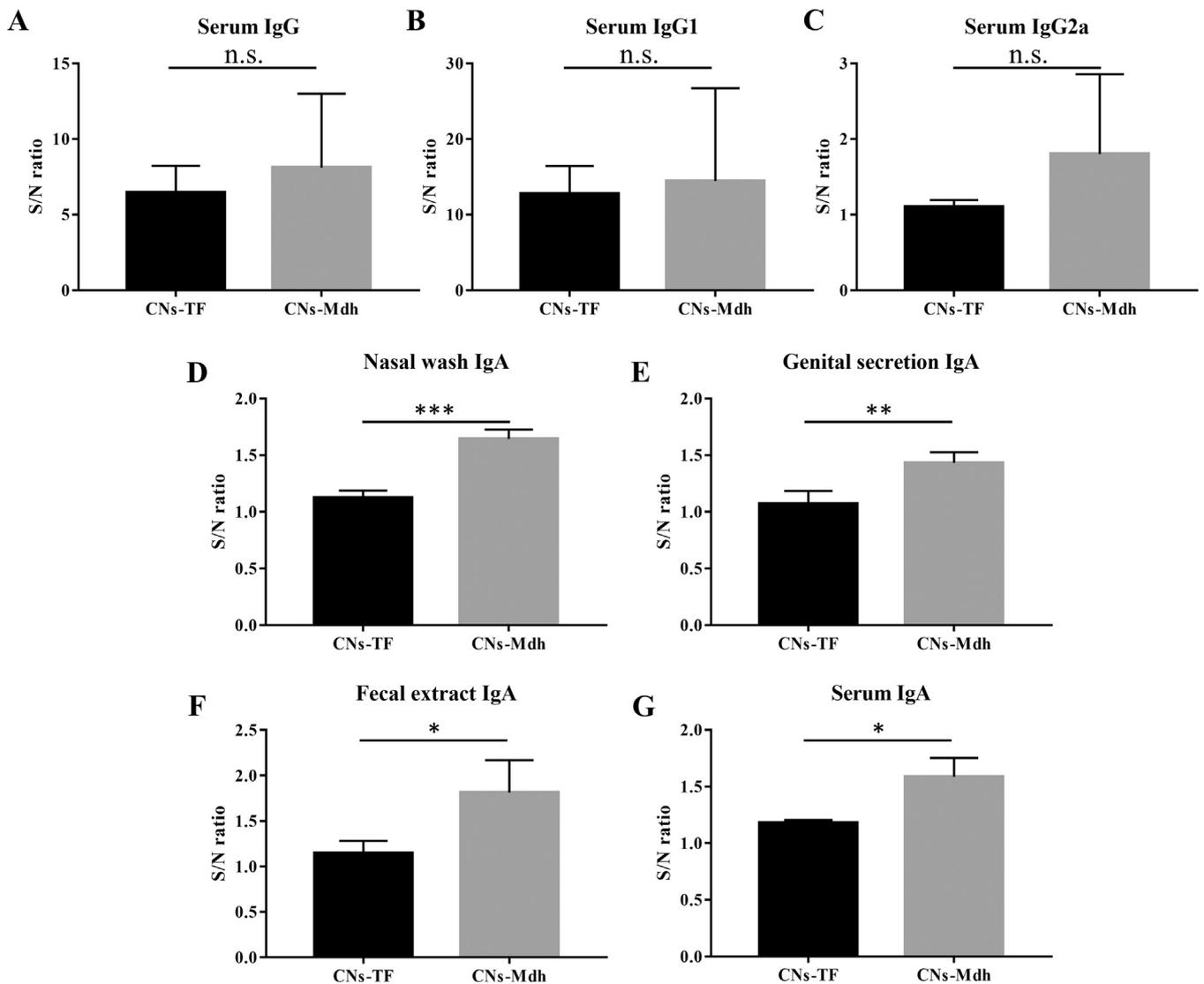
Fig. 7. Mdh-specific antibody measurements at 6 weeks after primary intranasal immunization. (A) Mdh specific total IgG, (B) IgG1 and (C) IgG2a in the sera of immunized mice. Secreted IgA and serum IgA were measured in mucosal samples and sera. Significant increase in the IgA levels of (D) nasal washes, (E) genital secretions, (F) fecal extracts and (G) sera were observed between the CNs-TF and CNs-Mdh-treated groups. The results are expressed as the sample to negative control ratio (S/N ratio) ± SD (n = 4), and statistical analysis was performed using Welch's t-test. Significant differences are indicated by [*]$P < 0.05$, [**]$P < 0.01$ and [***]$P < 0.001$; n.s.; not significant CNs-TF vs CNs-Mdh). *Footnotes:* CNs-TF, Trigger factor loaded chitosan nanoparticles; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.

microspheres <5 μm in diameter can be taken up by DCs and transferred to the spleen and lymph nodes to generate local and systemic immune responses [38]. The average size of nanoparticles in this study was less than 2 μm. Therefore, we can reasonably assume that our nanoparticles are able migrate to nearby lymph nodes through phagocytosis.

In vitro release tests of TF and Mdh showed similar release profiles from the CNs that are compared to those of previous studies [22,39,40]. The released protein is expected to act on the nasal mucosa both in solution and in particulate form. As seen from the confocal images in THP-1 cells, Mdh released from CNs, as well as Mdh alone stimulated cells, were well penetrated into the cells. The nuclear localization of Mdh protein was observed, and the intensity of protein in cytoplasm decreased with time. It means that the CNs loaded protein is transferred well into the cell and nucleus just as it is when the protein is stimulated alone. These results indicate that CNs used as delivery vesicles do not become an obstacle when proteins are delivered into cells. TNF-α and IL-6 are major inflammatory cytokines that can be used to evaluate

immune responses after antigen stimulation. THP-1 cells were differentiated into macrophages and stimulated with single proteins and protein-loaded CNs. In the cells treated with protein alone, significant differences in TNF-α and IL-6 were observed between Mdh and TF-treated cells at all time points, as has been previously reported [13]. Interestingly, unlike the protein-only stimulation, protein loaded CNs showed different TNF-α and IL-6 secretion patterns. TNF-α was lower in the protein-loaded CNs-treated cells than in the protein-treated cells. In Caco-2 cells, chitosan nanoparticles reduce the inflammatory response induced by LPS, i.e., TNF-α inhibition, through inhibition of the NF-κB pathway [23]. Furthermore, in RAW 264.7 macrophages, as the concentration of chitosan oligosaccharides increases, TNF-α tends to decrease, inhibiting the inflammatory effects of LPS [41]. Based on these results, the protein-loaded CNs-treated cells had decreased TNF-α production compared to protein-only stimulated cells due to the chitosan. Within the same context, TNF-α secretion in CNs-Mdh-treated cells was lower than that of CNs-TF-treated cells at 12 and 24 h because TF had a higher loading efficiency than Mdh
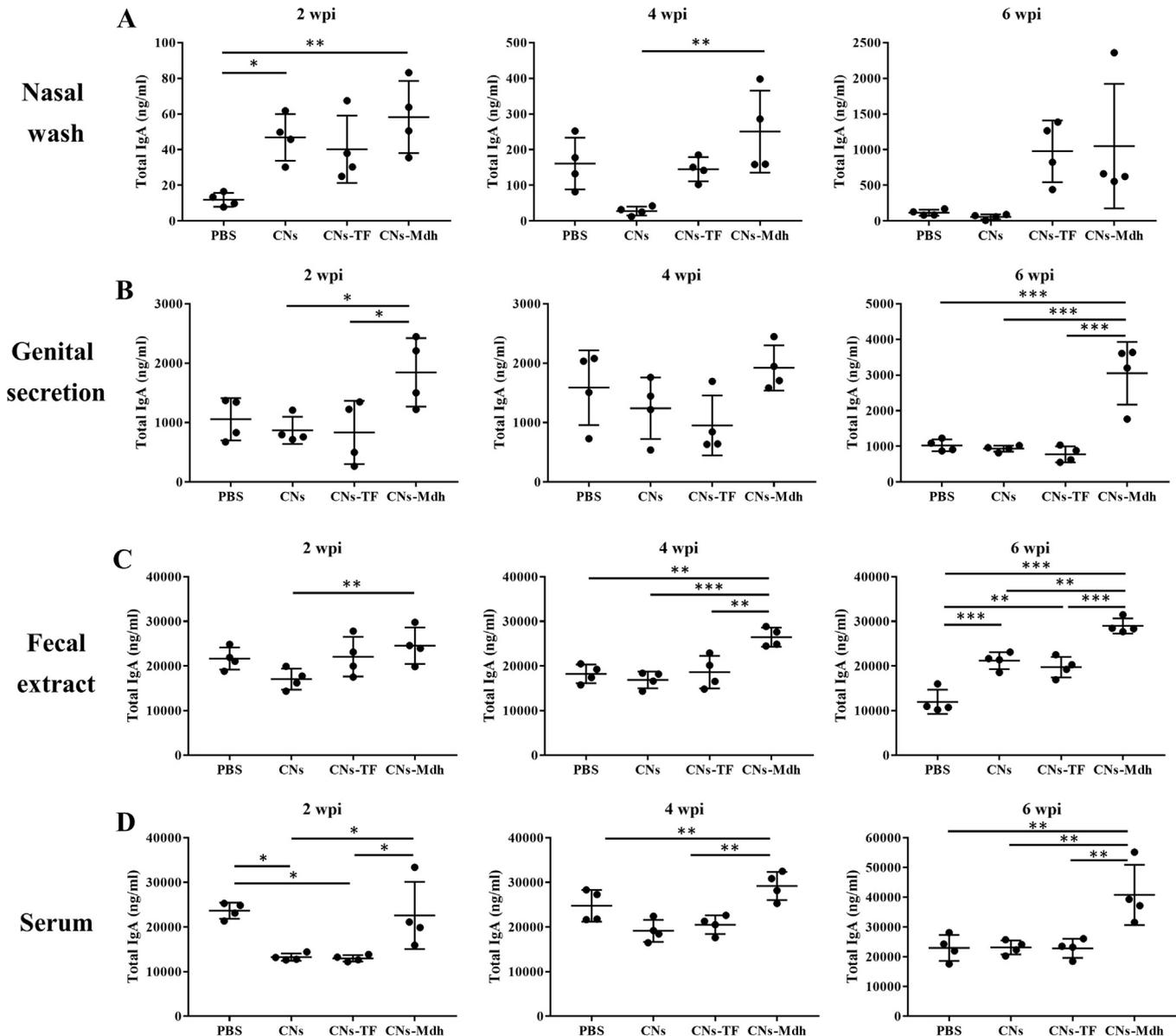
Fig. 8. Total IgA measurements in the nasal wash, genital secretion, fecal extract and serum samples at each time point (2, 4 and 6 weeks after primary immunization). Groups were statistically compared using one-way ANOVA with Tukey's post hoc multiple comparison test (*P < 0.05, **P < 0.01, ***P < 0.001). *Footnotes:* CNs, Chitosan nanoparticles; CNs-TF, Trigger factor loaded chitosan nanoparticles; CNs-Mdh, Malate dehydrogenase loaded chitosan nanoparticles.

(i.e., CNs-Mdh contained more CNs than CNs-TF). IL-6 can modulate the Th1/Th2 balance towards Th2 and was recently found to be associated with Th17 [42]. In the protein-loaded CNs-treated cells, IL-6 secretion was delayed more than in the protein-only treated cells, but it increased strongly at 24 h, at which time the IL-6 level of CNs-Mdh-treated cells was significantly higher than that of CNs-TF-treated cells. This IL-6 secretion pattern appears to be correlated with protein release from CNs. In the release test, protein was released slowly from CNs, delaying the immune response; however, an effective immune response was induced due to persistent stimulation.

For the *in vivo* studies, a significant increase in IgG-secreting cells was observed in the CNs-Mdh immunized mice (at 6 wpi), similar to Mdh-specific ELISA IgG, although there were no significant differences in the ELISA results. IL-4 secreting cells, which are involved in the Th2 immune response, were increased in the mouse splenocytes after 4 weeks of CNs-Mdh immunization. In addition, IgG1 was more predominant than IgG2a in specific

ELISAs, suggesting that CNs-Mdh induce a Th2-biased immune response. Mucosal secretions include IgA dimers and oligomers in both mice and humans. Generally, secreted IgA forms an IgA-antigen complex that binds to monocytes and macrophages through a low affinity receptor, FcαR1(CD89) [43]. Several cytokines associated with Th17 and Th2 immune response, including TGF-β, IL-4, IL-5, IL-6 and IL-10, stimulate secretory IgA production [44]. When IgA-opsonized particles bind to this receptor, opsonic action results, and it plays a critical role in antibody-mediated defense and the release of inflammatory mediators against infectious diseases [45], with its most important role being so-called immune exclusion, which prevents bacteria and viruses from attaching to epithelial surfaces [44]. IgA is an important component that reflects stimulation of the common mucosal immune system. In this study, specific IgA levels and total IgA levels were remarkably increased in the CNs-Mdh-immunized group compared to the CNs-TF-immunized group in all extracted samples except the total nasal wash IgA level at 6wpi. We sampled

representative mucosal effector sites from the respiratory, digestive and genital mucosa and found that intranasal immunization effectively induced IgA secretion in the digestive and genital mucosa as well as in the respiratory tract of mice. When stimulated by an antigen, IgA-producing B cells are produced and then circulate in the bloodstream, eventually settling at all of the mucosal effector sites [46]. According to this mechanism, intranasally immunized CNs-Mdh seems to stimulate the development of a systemic mucosal immune response through IgA production.

5. Conclusion

Chitosan nanoparticles loaded with recombinant *B. abortus* malate dehydrogenase produced a Th2-related immune response and simultaneously increase IgA production in mice. Furthermore, the CNs-Mdh delivered through the nasal cavity induced systemic and mucosal immune responses by increasing secretory IgA in the digestive and genital mucosa as well as the nasal mucosa and by inducing serum IgA elevation. Therefore, when CNs-Mdh induce effective mucosal immunity when immunized intranasally, they comprise a promising vaccine antigen and delivery system.

Acknowledgments

Declaration of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.005.

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