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REGULAR ARTICLES

Immunogenicity of adenovirus and DNA vaccines co-expressing P39 and lumazine synthase proteins of *Brucella abortus* in BALB/c mice

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Abstract

Brucella poses a great threat to animal and human health. Vaccination is the most promising strategy in the effort to control *Brucella abortus* (*B. abortus*) infection, but the currently used live vaccines interfere with diagnostic tests and could potentially result in disease outbreak. Therefore, new subunit vaccines and combined immunization strategies are currently under investigation. In this study, immunogenicity and protection ability of a recombinant adenovirus and plasmid DNA vaccine co-expressing P39 and lumazine synthase proteins of *B. abortus* were evaluated based on the construction of the two molecular vaccines. Four immunization strategies (single adenovirus, single DNA, adenovirus/DNA, DNA/adenovirus) were investigated. The results showed that the immunization strategy of DNA priming followed by adenovirus boosting induced robust humoral and cellular immune responses, and it significantly reduced the numbers of *B. abortus* in a mouse model. These results suggest that it could be a potential antigen candidate for development of a new subunit vaccine against *B. abortus* infection.

Keywords Adenovirus vaccine · BALB/c mice · *Brucella abortus* · DNA vaccine · Immunogenicity · Lumazine synthase · P39

Introduction

Brucellosis, caused by *Brucella* spp., is a highly infectious disease occurring in humans and various domestic and wild animals worldwide (Seleem et al. 2010; Walker and Blackburn 2015). This infection has wide-reaching economic effects and is a significant threat to global human health (Lopes et al. 2010; Godfroid et al. 2014; Vollmar et al. 2016). Although vaccination is among the most economic strategies, the current commercially available live vaccines can impede the detection of anti-*Brucella* antibodies conferred by wild strains (He and Xiang 2010; Christopher et al. 2010; Li et al. 2017). Thus, it is imperative to develop new diagnostic and preventative measures for the treatment of this disease.

Both DNA and live adenoviral vaccines offer an effect route for the activation of T helper (Th)1 and Th2 responses, and many studies have been reported on this work (Arévalo et al. 2009; Gabitzsch et al. 2011; Zhang and Zhou 2016; Tan et al. 2017). Presently, recombinant adenovirus expressing protective proteins of *Brucella* has not been reported, but some DNA vaccines related with *Brucella* have been studied (Luo et al. 2006; Jain et al. 2014; Golshani et al. 2015a). However, the results from these studies have demonstrated that single DNA vaccine confers a reduced antibody response and results in lower protection efficacy than the attenuated *Brucella* vaccines. Some strategies designed to improve the immune responses and protection efficacy of the DNA vaccines, including a combination of a DNA priming step and the homologous protein boosting, as well as co-expressing two proteins and the addition of adjuvant have been explored by others (Golshani et al. 2015a, b, 2016).

The P39 and lumazine synthase (LS) proteins are immunodominant and protective antigens conserved in animal *Brucella abortus* (*B. abortus*) (Luo et al. 2006; Tadepalli et al. 2016). The two proteins can elicit both humoral and cellular immune responses and are good candidates for use in future studies of vaccination against *B. abortus*.

In this study, the *P39* and *LS* genes were cloned into adenovirus serotype 5 vector, and co-expressed. Additionally a plasmid carrying both genes was also constructed using pcDNA3.1 vector. The immunogenicity and protection ability conferred by four immunization strategies (single adenovirus, single DNA, adenovirus/DNA and DNA/adenovirus) were

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evaluated. The results offer a new avenue for vaccine development.

Materials and methods

Mice and *Brucella*

Specific pathogen-free grade female BALB/c mice aged 6–8 weeks old were purchased from the Center of Experimental Animals, Lanzhou Institute of Biological Products (Lanzhou, China). *B. abortus* strain CVCC12 (Biovar II) was obtained from China Veterinary Culture Collection Center (CVCC) (Beijing, China) and proliferated as instructed.

Ad-P39/LS and pcDNA-P39/LS vaccines

Recombinant adenovirus Ad-P39/LS co-expressing *P39* and *LS* genes was propagated in HEK 293AD cells. Briefly, the *P39* and *LS* genes were inserted into the multiple cloning site I (MCS I) and MCS II of the pQCXIX retroviral vector, respectively. These cloning events generated the pQC-LL/BP, in which P39-IRES-LS fragment was cloned into transfer vector pShuttle-CMV. The resulting positive plasmid pShuttle-P39/LS was linearized with *Pme*I and transformed into *Escherichia coli* BJ5183 competent cells carrying pAdEasy-1 skeleton vector to obtain recombinant adenovirus pAd-P39/LS. The pAd-P39/LS was cleaved with *Pac* I to expose its inverted terminal repeats and transfected into HEK 293AD cells. Finally, Ad-P39/LS were generated and propagated in these cells.

The P39-IRES-LS fragment was cloned into pcDNA3.1 vector with *Not* I and *Xho*I to obtain plasmid pcDNA-P39/LS, and the plasmids were transfected into HEK 293AD cells and propagated.

All the above works were previously accomplished (not published), and the relevant results were not displayed in this study.

Immunization and sera collection

Six test groups of mice (10 per group) were immunized by bilateral intramuscular injection into the gastrocnemius and boosted two times with the same dose with a 2-week interval, except for the mice of group 5 which were immunized only once with attenuated vaccine A19 strain. The detailed immunization strategies are shown in Table 1.

Serum samples were collected from all mice of the six groups before immunization, and at 14 days after the first and second immunizations, respectively. Sera was stored at −20 °C until they were analyzed for specific antibodies. Pre-immune serum samples were used as negative controls.

Detection of antibodies

Analysis of antigen-specific IgG, IgG1, and IgG2a antibodies in serum samples were performed using indirect enzyme-linked immune-sorbent assay (ELISA) as previously described (Golshani et al. 2015a, b). Briefly, 96-well microtiter plates (Costar, Bethesda, MD, USA) were coated with 10 μg/ml ultrasonicated *B. abortus* A19 strain overnight in carbonate buffer (pH 9.6) at 4 °C. The plates were blocked with 1% BSA in PBS for 30 min at 37 °C. After thorough washing with PBST, the serum samples were added to the plate and allowed to incubate for 30 min at 37 °C. The plates were washed again and were reacted with HRP-labeled anti-mouse IgG, IgG1, and IgG2a diluted in PBST at 1:1000 for 30 min at 37 °C. Plates were washed and developed with TMB, while being kept in a dark place for 10 min. Finally, stop solution was added, and optical density (OD) values were immediately measured at 450 nm using an ELISA reader. All samples were run in triplicate. The cutoff value for the assay was calculated as the mean specific OD plus 3 standard deviation (SD) for 30 pre-immunization serum samples.

Lymphoproliferation assay

The procedure was performed as in a previous report (Golshani et al. 2016). Two weeks after the second immunization, mice spleens were removed (five mice/group) and ground under sterile conditions using a 5-ml syringe plunger, and single-cell suspensions were obtained by filtration through stainless steel mesh. Splenocytes were isolated by mouse lymphoprep (Dakewe, Shenzhen, China) and placed into 96-well-plate with 100 μl/well at a density of 5×10^6 cells/ml in complete medium (RPMI 1640 + 10% FBS + 100 U/ml penicillin/streptomycin). Cells were incubated with 5 μg/ml *B. abortus* A19 strain (10 μg/ml) or concanavalin A (5 μg/ml) or medium alone (negative control) in a 5% CO_2 humidified incubator at 37 °C. The proliferative activity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml, Sigma) dye assay. The stimulation index (SI) was calculated as the ratio of the average OD_{570} of antigen-stimulated cells to the average OD_{570} of unstimulated cells.

Flow cytometric analysis of surface markers of lymphocytes

For flow assays, 2 ml of the splenocyte suspension (5×10^6 cells/ml) was centrifuged for 5 min at 2000 rpm, and the supernatants were discarded. The cells were washed one time with 1 ml fluorescence solution, and the supernatant was discarded. Then, the cells were suspended with 30 μl fluorescence solution. For each tube, 1 μl PerCP-CD^{3e}, 1 μl PE-CD^{8a}, and 0.5 μl FITC-CD^{4+} were added and mixed except

Table 1 Immunization strategies for six test groups

Groups	Numbers of mice	Vaccines	First immunization (each mouse)	Second immunization (each mouse)
1	10	PBS	100 μl	100 μl
2	10	pcDNA-P39/LS	50 μg	50 μg
3	10	Ad-P39/LS	100 $TCID_{50}$	100 $TCID_{50}$
4	10	Ad-P39/LS and pcDNA-P39/LS	100 $TCID_{50}$ of Ad-P39/LS	50 μg of pcDNA-P39/LS
5	10	pcDNA-P39/LS and Ad-P39/L	50 μg of pcDNA-P39/LS	100 $TCID_{50}$ of Ad-P39/LS
6	10	A19	5×10^8 CFU	Not

"Not" represented that the second immunization was not carried out

CFU colony-forming unit

for the control tube. The mixtures were incubated for 45 min in the dark and were then washed three times in the fluorescence solution. Finally, the cells were suspended in 200 μl fluorescence solution and filtered into a flow cytometry tube through a nylon membrane. The cells were then analyzed using a flow cytometer.

Cytokine assay

Splenocyte suspensions (5×10^6 cells/ml) were placed into a 24-well plate with 2 ml/well in duplicate. The fractions of A19 strain (1×10^8 cells/ml) treated with ultrasonication were placed into the plate at 10 μl/well. The plate was incubated for 120 h at 37 °C with 5% CO_2. The supernatant of each well was collected for detection of IL-10 and IL-12.

Protection experiment

The experiment was performed in a BSL-3 laboratory as recommended (Golshani et al. 2016). Fourteen days after the second vaccination, five mice of each group were challenged by intraperitoneal route with 1×10^5 colony-forming unit (CFU) of *B. abortus* strain CVCC12 in 100 μl of PBS. At 4 weeks, post-challenge, spleens of the mice were aseptically removed and weighed. Each spleen was homogenized in PBS with 1:10 (g/ml, *w*/*v*) and serially diluted tenfold. Each dilution was applied to *Brucella* agar to determine the CFU. The results are presented using the mean ± SD of Log_{10}^{CFU} per group. Log units of protection were obtained by subtracting the mean Log_{10}^{CFU} of the vaccinated group from the mean Log_{10}^{CFU} of the PBS control group. Log units of protection should increase following reduction of the bacteria.

Statistical analysis

One-way analysis of variance was carried out to analyze the differences between the groups using SPSS 20.0. Statistical significance was assumed at the $p < 0.05$ level.

Results

Detection of antibodies

For group 1, the levels of IgG, IgG1, and IgG2a antibodies did not change during the whole process ($p > 0.05$). For group 6, the three antibodies rapidly rose after the first immunization and kept high levels during the process.

The levels of IgG in the mice from groups 2 to 5 rose after the first immunization and significantly increased again after the second immunization. The IgG levels in group 5 was the highest, taking turn groups 4, 3 and 2. The OD_{450} values based on the specific IgG in all groups were shown in Fig. 1a.

For groups 2 to 5, the levels of IgG1 and IgG2a antibodies after the first immunization had no significant differences when compared with those before immunization ($p > 0.05$). The two antibodies significantly rose after the second immunization. IgG2a antibodies were significantly higher compared with IgG1 in each corresponding group ($p < 0.05$). OD_{450} values based specific IgG1 and IgG2a in all groups are shown in Fig. 1b, c, respectively.

Lymphoproliferation assay

Both strain A19 and ConA promote the proliferation of splenocyte T cells derived from the mice in groups 2 to 6, but no proliferation was observed in group 1. The SIs based-ConA were slightly higher than the values based-A19 in each corresponding group. The SIs from group 6 were the highest, followed by groups 5, 4, 3, and 2. The values from groups 5 and 4 were different from those in groups 3 and 2 ($p < 0.05$). No significant differences were observed between groups 5 and 4 and groups 3 and 2. ($p > 0.05$). These results are shown in Fig. 2.

Flow cytometric analysis of surface markers of lymphocytes

The percentages of CD^{3+} and CD^{4+} T cells from groups 2 to 6 were significantly high compared with those from group 1

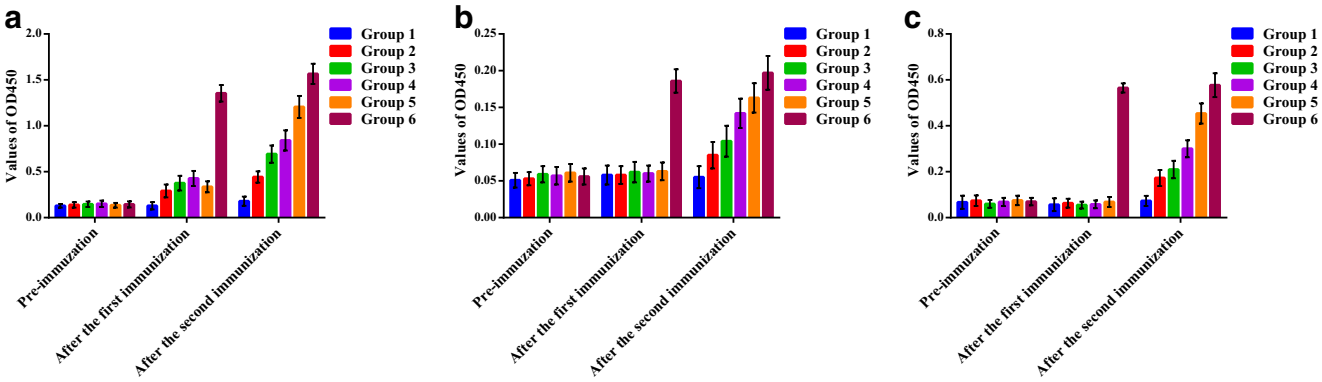

Fig. 1 The levels of IgG (**a**), IgG1 (**b**), and IgG2a (**c**) derived from the immunized and control mice evaluated by indirect ELISA

($p < 0.05$). Although the percentages in group 6 were the highest, no significant differences were observed between groups 5 and 6 ($p > 0.05$). The percentages had no differences between groups 2 and 3 ($p > 0.05$). The percentages from group 4 were slightly high than those from groups 2 and 3. For CD^{8+}, no statistically significant differences were obtained in groups 1 to 5 ($p > 0.05$), but the value in group 6 was significantly higher compared with those from the other five groups ($p < 0.05$). These results are displayed in Fig. 3.

Cytokine assay

No statistically significant differences in IL-10 were observed between groups 1 to 5 ($p > 0.05$), but the level in group 6 was significantly higher in comparison with those from the other five groups ($p < 0.05$). For IL-12, the levels in groups 2 to 6 were significantly higher than group 1. The value from group 6 was highest followed by groups 5 and 4 ($p < 0.05$), with no difference in induced levels between groups 4 and 5, although they were elevated in these groups when compared with groups 2 and 3 which also had no major differences in induction of this cytokine ($p > 0.05$). The values for this analysis are shown in Fig. 4.

Protection experiment

Protection was determined as a significant reduction in the number of bacteria in the spleens from immunized mice compared to the mice which received PBS. For group 5, the combined vaccines conferred significant protection with the log unit of 1.35, which was similar to that achieved by the live attenuated A19 vaccine. The immunization strategy in group 4 also revealed protection ability with a log unit of 1.16. The levels of protection were lower with a single vaccine approach as seen in groups 2 and 3. The detailed results are displayed in Table 2.

Discussion

Brucella is an intracellular pathogen, so it is very difficult to eradicate *Brucella* infection with antimicrobial agents. Vaccination is the most promising strategy to control the disease, but the currently used live vaccines interfere with diagnostic tests and have the potential risk of spreading the disease if the attenuation is incomplete (Christopher et al. 2010; Wang et al. 2015). In order to avoid the disadvantages, some new strategies including subunit recombinant protein vaccines (Du

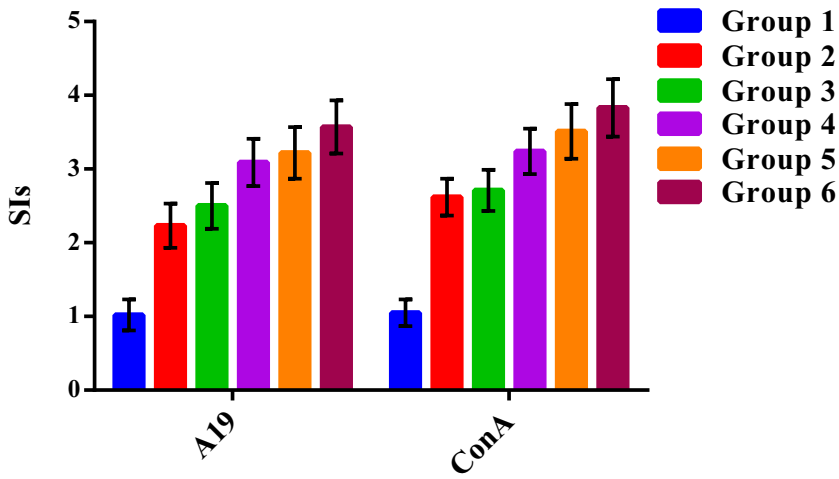

Fig. 2 Proliferative responses of lympho-splenocytes derived from the immunized and control mice against A19 antigen and ConA stimulation

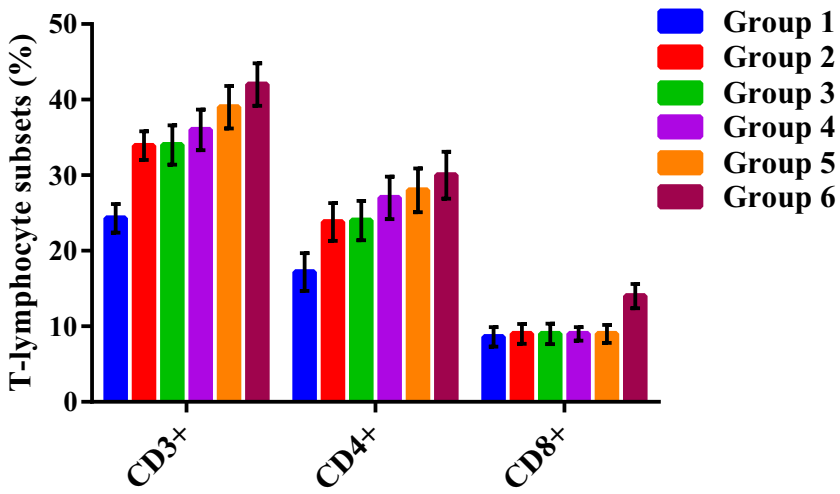

Fig. 3 Analysis of spleen T lymphocyte subsets by FCM

et al. 2016), vector vaccines based on *E. coli* (Gupta et al. 2012), *Salmonella enterica* (Zhao et al. 2009), *Salmonella typhimurium* (Kim et al. 2016), influenza viruses (Tabynov et al. 2014), and plasmid DNA vaccines (Luo et al. 2006; Cassataro et al. 2007; Jain et al. 2014) have all been evaluated for the prevention of *Brucella* infections. Presently, studies on recombinant adenovirus vaccines against some viruses and bacteria have been reported (Zhang and Zhou 2016; Tan et al. 2017), but the relevant research for *Brucella* has not been reported.

Moreover, some studies reveal that combined immunization with multivalent vaccines can elicit stronger immune responses and better protection against *Brucella* than the relevant univalent vaccines in mice model. *Brucella* Omp2b protein administered as Pro/Pro, DNA/DNA, or DNA/Pro regimen is capable of inducing vigorous humoral and cellular responses (Golshani et al. 2016). A recombinant fusion protein (rL7/L12-TOmp31) provided significant protection levels against *Brucella melitensis* (*B. melitensis*) and *B. abortus* challenges (Golshani et al. 2015a). The pcDNA-L7/L12-TOmp31 priming followed by rL7/L12-TOmp31 protein boosting led to improved protection against *B. abortus* or *B. melitensis* infection (Golshani et al. 2015b). A divalent genetic vaccine based on the L7/L12-Omp16 or L7/L12-P39 can elicit a stronger cellular immune response and better immunoprotection against *B. abortus* in comparison to single proteins (Luo et al. 2006). A 27-amino acid epitope derived from Omp31induced peptide- and BLS-specific Th1 and cytotoxic T responses (Cassataro et al. 2007).These above studies showed that divalent genetic vaccine encoding two proteins or agenetic vaccine priming and recombinant protein boosting could elicit stronger immune responses and provide protection against *B. melitensis* and *B. abortus* infection in BALB/c mice. Based on these works, we wanted to evaluate the immunogenicity and protection ability of recombinant adenovirus and plasmid DNA vaccines co-expressing P39 and LS proteins of *B. abortus* in BALB/c mice model. Furthermore, four immunization strategies (single adenovirus,

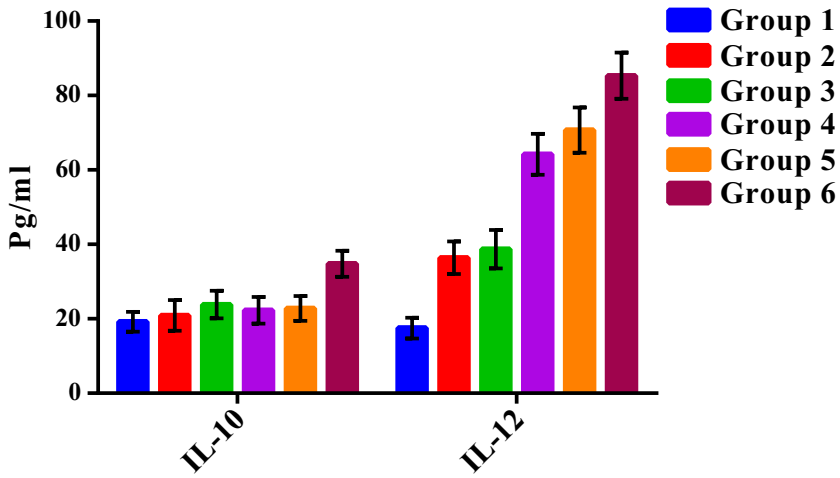

Fig. 4 The levels of IL-10 and IL-12 derived from the immunized and control mice

Table 2 Protection against *B. abortus* CVCC12 infection induced by vaccination

Groups ($n = 5$)	Vaccine or control	$\log_{10}$ CFU of CVCC12 in spleen (mean ± SD)	Log units of protection in spleen
1	PBS	5.90 ± 0.12	0
2	pcDNA-P39/LS	4.95 ± 0.14*	0.95
3	Ad-P39/LS	4.91 ± 0.15*	0.99
4	Ad-P39/LS and pcDNA-P39/LS	4.74 ± 0.16*	1.16
5	pcDNA-P39/LS and Ad-P39/L	4.55 ± 0.16*	1.35
6	A19	4.40 ± 0.13*	1.50

*Significantly different compared to control group ($p < 0.05$)

single DNA, adenovirus/DNA, and DNA/adenovirus) were carried out. Since immunity against *Brucella* requires cell-mediated mechanisms (Th1 response), the antibody isotypes, splenocyte proliferative responses, T lymphocyte subsets, and the cytokines produced after immunization were evaluated.

In order to understand the humoral response, IgG levels of the mice were detected in this work. Th1 cells mainly mediate cellular immunity to accelerate IgG2a antibody (Carmi et al. 2015; Golshani et al. 2015b; Im et al. 2016), and IgG2a has the major role in immunity against *Brucella* by facilitating phagocytes. Therefore, both IgG1 and IgG2a isotypes were also done. After the second vaccination, IgG, IgG1, and IgG2a antibodies rapidly rose in all immunized mice except for the animals treated with PBS. The levels of IgG were the highest, followed by IgG2a and IgG1, and IgG2a exhibited dominance over IgG1. The pcDNA-P39/LS priming followed by Ad-P39/LS boosting (group 5) regimen induced higher antibody levels in comparison to single vaccines (groups 2 and 3) or Ad-P39/LS priming followed by pcDNA-P39/LS boosting strategy (group 4). The mice immunized with A19 (group 6) still exhibited the highest levels of antibody stimulation. These results indicated that pcDNA-P39/LS or Ad-P39/LS vaccines can elicit mixed Th1/Th2 type responses and Th1 response exhibited dominance.

In order to study cell-mediated immune response generated by pcDNA-P39/LS and Ad-P39/LS vaccines, splenocytes of mice from groups 1 to 6 were stimulated with A19 antigen or ConA. Based on the SIs, the splenocytes of all immunized mice showed significant proliferation than PBS control animals. The mice primed with pcDNA-P39/LS and boosted with Ad-P39/LS (group 5) were elicited better cellular immune response than those from other immunized mice, except the mice vaccinated with A19 antigen (group 6).

Since the cellular arm of the Th1 response is essential for conferring protection against *Brucella* infection (Jain et al. 2014), the splenocyte proliferative responses were evaluated. T cell subsets consisting of CD3+, CD4+, and CD8+ which produces Th1 or Th2 type cytokines were detected by FCM analysis. The results showed that CD^{3+} and CD^{4+} T cells from immunized mice rose significantly in comparison to PBS control animals. CD^{8+} cells had no changes among PBS control, pcDNA-P39/LS, Ad-P39/LS, Ad-P39/LS and pcDNA-P39/LS, and pcDNA-P39/LS and Ad-P39/L groups, but CD^{8+} cells from the mice (group 6) vaccinated with A19 had significant increase when compared with other groups. In addition, the percentages of CD^{3+} and CD^{4+}T cells had no statistic differences between pcDNA-P39/LS and Ad-P39/L group and A19 group, although the values from A19 group were slightly higher than those from pcDNA-P39/LS and Ad-P39/L group. These results indicated that splenocytes from mice vaccinated with pcDNA-P39/LS or Ad-P39/LS vaccines were able to proliferate, and the two vaccines could induce mixed Th1/Th2-type responses.

To further understand the immune response, the cytokines IL-12 (Th1-type cytokine) and IL-10 (Th2-type cytokine) were tested, and Th1-type immune response in the form of high levels of IL-12 in all vaccinated groups was observed. Two step immunization strategies (groups 5 and 4) induced higher IL-12 levels in comparison to pcDNA-P39/LS or Ad-P39/LS regimen alone. The levels of IL-10 in all mice except for the animals in group 6 showed no significant changes. These results demonstrated that pcDNA-P39/LS priming followed by Ad-P39/LS boosting regimen could induce mainly Th1 type immune response.

Protection experiments were carried out to evaluate protective ability of the pcDNA-P39/LS or Ad-P39/LS vaccines. All the regimens using pcDNA-P39/LS or Ad-P39/LS vaccines could reduce the numbers of *B. abortus* CVCC12 strain in the spleens from the immunized mice. A combination of pcDNA-P39/LS priming with Ad-P39/LS boosting (group 5) significantly reduced the numbers of *B. abortus* CVCC12 strain when compared to the other three immunization strategies (groups 2, 3, and 4).

In conclusion, the current study demonstrated that pcDNA-P39/LS priming with Ad-P39/LS boosting regimen could effectively elicit robust humoral and cellular immune response and significantly reduce the numbers of *B. abortus* CVCC12 strain in BALB/c mice. This could be a potential antigen candidate following further studies in relevant target species.

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Compliance with ethical standards

The study protocol was approved by the Animal Care and Use Committee of Life Science and Engineering College, Northwest University for Nationalities.

Conflict of interest The authors declare that they have no conflict of interest.

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