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Immunization with a combination of recombinant *Brucella abortus* proteins induces T helper immune response and confers protection against wild-type challenge in BALB/c mice

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Summary

Protective efficiency of a combination of four recombinant *Brucella abortus* (*B. abortus*) proteins, namely, ribosomal protein L7/L12, outer membrane protein (OMP) 22, OMP25 and OMP31, was evaluated as a combined subunit vaccine (CSV) against *B. abortus* infection in RAW 264.7 cell line and murine model. Four proteins were cloned, expressed and purified, and their immunocompetence was analysed. BALB/c mice were immunized subcutaneously with single subunit vaccines (SSVs) or CSV. Cellular and humoral immune responses were determined by ELISA. Results of immunoreactivity showed that these four recombinant proteins reacted with *Brucella*-positive serum individually but not with *Brucella*-negative serum. A massive production of IFN-γ and IL-2 but low degree of IL-10 was observed in mice immunized with SSVs or CSV. In addition, the titres of IgG2a were heightened compared with IgG1 in SSV- or CSV-immunized mice, which indicated that SSVs and CSV induced a typical T-helper-1-dominated immune response *in vivo*. Further investigation of the CSV showed a superior protective effect in mice against brucellosis. The protection level induced by CSV was significantly higher than that induced by SSVs, which was not significantly different compared with a group immunized with RB51. Collectively, these antigens of *Brucella* could be potential candidates to develop subunit vaccines, and the CSV used in this study could be a potential candidate therapy for the prevention of brucellosis.

Introduction

Brucella spp. are Gram-negative, facultative intracellular, non-motile and non-spore forming pathogens that cause brucellosis in humans and animals worldwide, which threaten their health (Gheibi *et al*., 2018). Brucellosis has caused huge economic losses to developing countries (Yagupsky and Baron, 2005). The genus *Brucella* has been classified into eleven species based on its primary preferred host, such as *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, *B. microti*, *B. papionis*, *B. pinnipedialis*, *B. ceti* and *B. inopinata* (Moreno, 2014). *Brucella* infections induce various clinical symptoms in animals and humans, for example, recurrent fever, arthritis, neurological symptoms and infertility or abortion (Atluri *et al*., 2011).

Vaccine immunization is the most efficient mean for prevention and control of animal brucellosis (Avila-Calderon *et al*., 2013). Subunit vaccine is the hot spot in the research and development of *Brucella* vaccine. Subunit vaccines do not use living *Brucella*, thereby eliminating safety (Pasquevich *et al*., 2011). Subunit vaccines are safe for animals because they cannot cause abortion. In general, purified proteins as subunit vaccines can stimulate immune responses in animals. Consequently, many protective antigens for brucellosis have been used to develop single subunit vaccines (SSVs). More studies have been reported on the protective efficiency of recombinant *Brucella* proteins as subunit vaccine against *Brucella* infection. These single SSVs include outer membrane protein (OMP) 10 (Im *et al*.,

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2018; Shim *et al*., 2020), OMP19 (Im *et al*., 2018; Shim *et al*., 2020), OMP28 (Im *et al*., 2018), BP26 (Gupta *et al*., 2019), 50S ribosomal protein L7/L12 (Gupta *et al*., 2019), VIRB10 (Pathak *et al*., 2018), BCSP31 (Xu *et al*., 2019) and P39 (Tadepalli *et al*., 2017). Based on previous results, combined subunit vaccine (CSV) using more than two recombinant proteins could confer higher potential immune response against *Brucella* infection than SSV. These CSVs include L7/L12-SOmp2b (Golshani *et al*., 2018a,b), L7/L12-TOmp31-SOmp2b (Golshani *et al*., 2018a,b), L7/L12-Omp25 (Paul *et al*., 2018; Gupta *et al*., 2020) and Adk-SecB (Huy *et al*., 2020a,b). Amongst these CSVs, recombinant proteins L7/L12 and OMPs are considered as potential immunogens, which can induce strong protective effects against *Brucella* infection. Therefore, in this study, we evaluated the ability of a combination of four *B. abortus* recombinant proteins, namely, L7/L12, OMP22, OMP25 and OMP31, as SSVs or CSV to induce immune response against *B. abortus* infection in BALB/c mice.

OMPs are integral proteins that are located on the outer membrane of *Brucella*. The weight and size of these OMPs are different. They are divided into three groups (Verstreate *et al*., 1982). L7/L12, OMP22, OMP25 and OMP31 belong to the third group (Cloeckaert *et al*., 2002). OMP22 name refers to its calculated mass of 22 kDa but was called previously OMP3b (Guzman-Verri *et al*., 2002). The antigenic protein OMP22 plays an essential role in the pathogenicity of *Brucella* (Cloeckaert *et al*., 2002; Saadi *et al*., 2017). OMP25 is conserved amongst the *Brucella* species. OMP25 mutant strain of *Brucella* is shown to be attenuated in mice emphasizing on the role of OMP25 in *Brucella* virulence (Edmonds *et al*., 2002). OMP25 could induce Th1- and Th2-type immune responses (Goel and Bhatnagar, 2012). In *Brucella*-infected macrophages, OMP25 could inhibit TNF-α production (Jubier-Maurin *et al*., 2001). OMP31 antigen is an essential protein of *Brucella*, which is a critical factor for bacterial pathogenicity (Vishnu *et al*., 2017; Shirdast *et al*., 2021). The recombinant protein L7/L12 was considered as a potential immunogen and demonstrated to induce strong protective effects against *Brucella* infection (Oliveira and Splitter, 1994; Golshani *et al*., 2015). Thus, in the present study, we assessed the ability of purified recombinant proteins, including L7/L12, OMP22, OMP25 and OMP31, to prevent brucellosis when administered to BALB/c mice alone or in combination.

Results

Purification and immunoreactivity of recombinant proteins

The four genes encoded for L7/L12, OMP22, OMP25 and OMP31 were cloned, expressed and purified. Using SDS-PAGE and Coomassie brilliant blue staining, the target molecular masses for rL7/L12, rOMP22, rOMP25 and rOMP31 were approximately 77.55, 82.38, 82.22 and 87.56 kDa respectively (Fig. 1A). Meanwhile, the immunoreactivity of these purified proteins was evaluated by Western blot assay, and the results indicated that all purified proteins strongly reacted with S2308-vaccinated mouse serum (Fig. 1B).

Humoral immunoreaction analysis by inducing IgG1 and IgG2a antibodies in mice immunized with SSVs and CSV

In this study, sera of mice were collected at week 8 after the last immunization for subsequent immunological analyses. ELISA was utilized to measure the presence of SSV- and CSV-specific IgG1 and IgG2a antibodies in serum samples. The results showed that after completing the immunization, SSVs and CSV induced significantly higher humoral immunity than the PBS and adjuvant-immunized groups, whereas no difference in the humoral immune response was observed between the PBS and adjuvant-immunized groups (Fig. 2). In the rL7/L12, rOMP22, rOMP25 and rOMP31 groups, the IgG2a/IgG1 ratio was 1.03, 1.06, 1.06 and 1.07 respectively (Fig. 2). The CSV group induced the highest IgG1 and IgG2a production in which the IgG2a/IgG1 ratio was 1.15. Furthermore, the titres of IgG2a induced by these purified proteins were higher than those of IgG1,

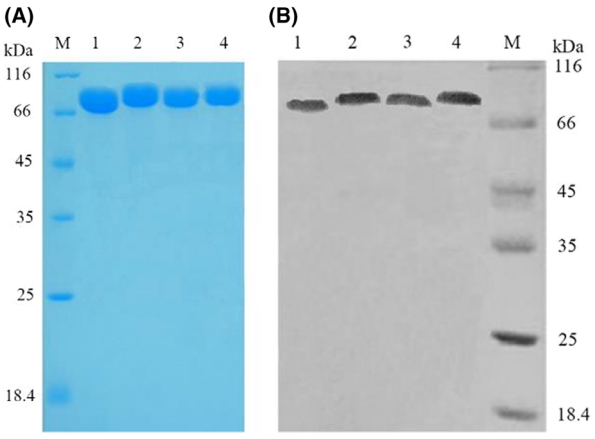

Fig. 1. SDS-PAGE identification and Western blot analysis of the recombinant proteins. (A) SDS-PAGE identification of the rL7/L12 (77.55 kDa), rOMP22 (82.38 kDa), rOMP25 (82.22 kDa) and rOMP31 (87.56 kDa). Lanes: M, protein marker; 1, expression product of rL7/L12; 2, expression product of rOMP22; 3, expression product of rOMP25; 4, expression product of rOMP31. (B) Western blot identification of the recombinant proteins using mouse anti-rL7/L12 polyclonal antibody, mouse anti-rOMP22 polyclonal antibody, mouse anti-rOMP25 polyclonal antibody and mouse anti-rOMP31 polyclonal antibody. Lanes: 1, expression products of rL7/L12; 2, expression product of rOMP22; 3, expression product of rOMP25; 4, expression product of rOMP31; M, protein marker.

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indicating that these proteins induced a Th1-dominant immune response in mice.

Production of cytokines in mice immunized with SSVs and CSV

The presence of cytokines in sera was assessed by ELISA to investigate the cell-mediated immune response. Sera were collected from all mice at week 8 after the last immunization. The secretion of IFN-γ and IL-2 in the SSV and CSV groups was significantly increased compared with that of the PBS or adjuvant control group ($P < 0.01$, Fig. 3A and B). The CSV group displayed considerably increased IFN-γ production by 2.41-fold and IL-2 production by 2.88-fold compared with PBS (Fig. 3A and B). However, the RB51 group remarkably induced the highest IFN-γ and IL-2 levels, which play an important role in resisting against *Brucella* infection (Fig. 3A and B). By contrast, IL-10 production was decreased in all experimental groups compared with the PBS or adjuvant group (Fig. 3C). The CSV group showed decreased IL-10 production by 1.85-fold compared with PBS (Fig. 3C). In addition, the CSV group induced higher IFN-γ and IL-2 levels than IL-10 levels of approximately 1.75-fold and 1.58-fold, respectively, compared with PBS. The IFN-γ and IL-2 levels induced by SSVs and CSV were significantly higher than the IL-10 level, thereby suggesting that SSVs and CSV induced a Th1-dominant immune response in mice. Moreover, the production of proinflammatory cytokines (TNF-α, IL-1β and IL-6) was significantly increased in the SSV and CSV groups compared with the PBS or adjuvant group (Fig. 3D–F). In particular, CSV-immunized mice produced 2.16-, 1.95- and 4.72-fold increases in the levels of TNF-α, IL-1β and IL-6 proinflammatory cytokines, respectively, compared with the PBS group.

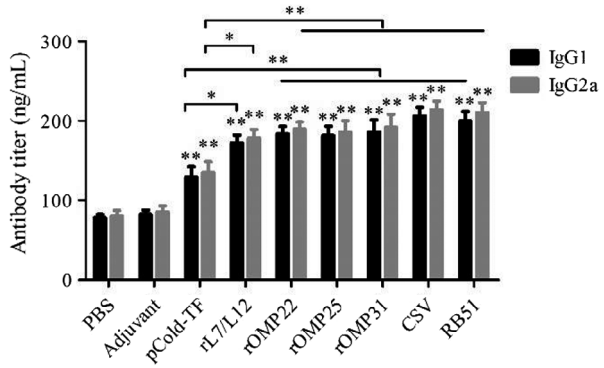

Fig. 2. IgG1 and IgG2a antibody production in the sera of mice immunized with different vaccines. BALB/c mice were immunized with purified rL7/L12, rOMP22, rOMP25 and rOMP31 for SSVs, CSV, PBS, adjuvant or pCold-TF at weeks 0, 2, 4 and 6. As another positive control, mice were immunized with 1×10^6 CFU of RB51 at day 0. Serum samples were obtained from tail veins at week 8 after the last immunization. IgG1 and IgG2a antibody titre was determined by ELISA. The data are presented as the means ± SD ($n = 5$ per group). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

In addition, the levels of IFN-γ, IL-2, IL-10, TNF-α, IL-1β and IL-6 cytokines in the splenocytes of the immunized mice at week 8 after the last immunization were detected. The splenocytes of vaccinated mice were stimulated with heat-killed S2308, ConA (positive control), or complete RPMI 1640 medium (negative control). When stimulated with heat-killed S2308, spleen cells from SSVs, CSV and RB51 produced significantly higher amounts of IFN-γ, IL-2, TNF-α, IL-1β and IL-6 relative to that of PBS- or adjuvant-dosed mice ($P < 0.01$, Fig. 4). By contrast, IL-10 production was decreased in all experimental groups compared with the PBS or adjuvant group (Fig. 4C). ConA stimulation induced the production of all cytokines in splenocytes from all groups, whereas no cytokine production was induced by RPMI 1640 medium (Fig. 4). Except for IL-10, no cytokine production was induced by PBS or adjuvant stimulation in any of the groups (Fig. 4). However, no statistically significant difference in the production of IFN-γ, IL-2, IL-10, TNF-α, IL-1β and IL-6 was observed between SSVs or CSV and RB51. Collectively, these results indicated that immunization with SSVs and CSV could induce cellular immune response.

Measurement of lymphocyte proliferation

Lymphocyte proliferation ratios were detected to assess cellular immunity. At week 8 after the last immunization, lymphocyte transformation rates in immunized mice were significantly higher than those in the PBS or adjuvant control groups ($P < 0.01$, Fig. 5). Although the lymphocyte proliferation ratios of the live vaccine group (RB51) were slightly higher than those of the SSV and CSV groups, no significant differences were observed ($P > 0.05$, Fig. 5). The lymphocyte proliferation ratios in the SSV groups were slightly lower than those in the CSV group, but the difference was not significant ($P > 0.05$). These results indicated that SSVs and CSV significantly promoted cellular immune responses.

Cytokine production in RAW 264.7 cells

Macrophages have been shown to constitute an important site for *Brucella* intracellular replication within tissues. Here, cytokine production from cell culture supernatant was measured using ELISA. Significantly higher IFN-γ, IL-2 and IL-12p70 production levels were observed in SSV- and CSV-treated cells than in PBS-treated ones ($P < 0.01$), and this difference increased with time (Fig. 6A–C). By contrast, the IL-10 level was

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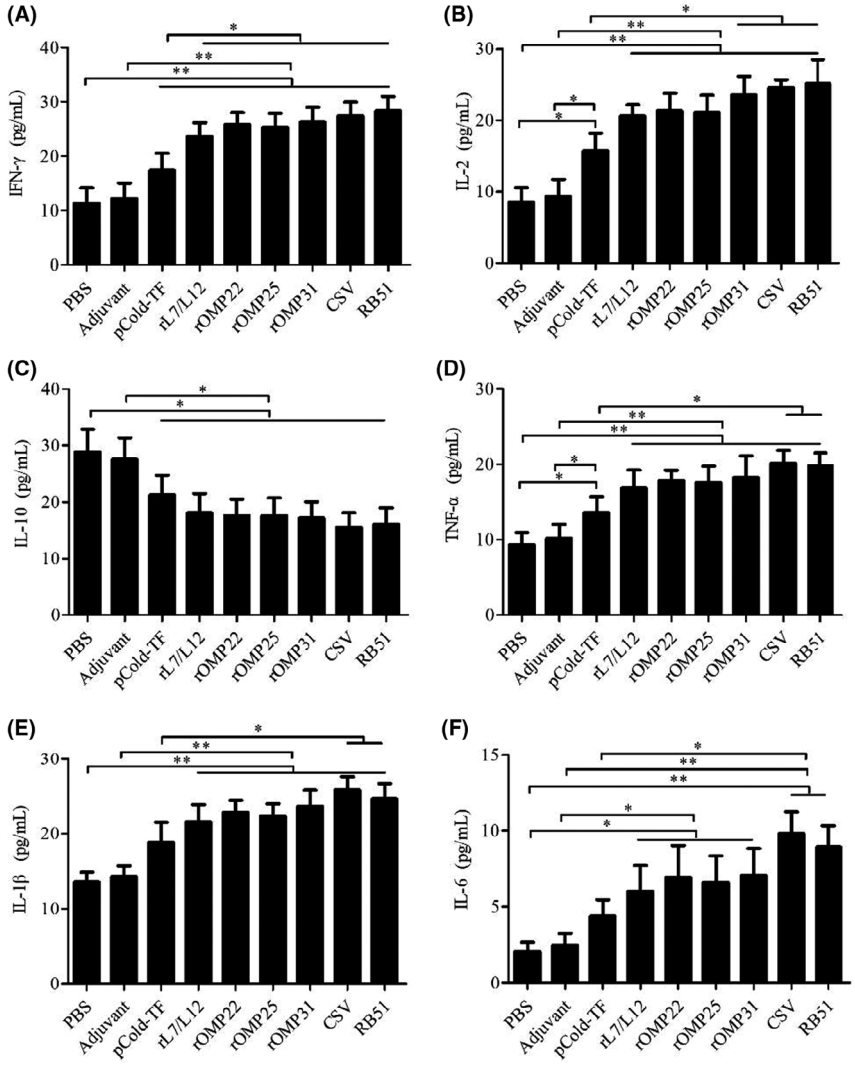

Fig. 3. Cytokine concentration in the sera of immunized mice. BALB/c mice were immunized with purified rL7/L12, rOMP22, rOMP25 and rOMP31 for SSVs, CSV, PBS or adjuvant, respectively, at weeks 0, 2, 4 and 6. As another positive control, mice were immunized with 1×10^6 CFU of RB51 at day 0. Serum samples were obtained from tail veins at week 8 after the last immunization. Concentration of IFN-γ (A), IL-2 (B), IL-10 (C), TNF-α (D), IL-1β (E) and IL-6 (F) was analysed by ELISA. The data are presented as the means ± SD ($n = 5$ per group). Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

decreased in SSV- and CSV-treated cells (Fig. 6D). CSV-treated cells significantly induced 1.38-fold higher in IFN-γ, 1.38-fold higher in IL-2 and 1.36-fold higher in IL-12p70 production at 12 h post-infection and continuously increased at 24 and 48 h. However, the IL-10 level showed a 3.45-fold, 4.21-fold and 6.09-fold decrease at 12, 24 and 48 h post-infection, compared with PBS-treated cells respectively (Fig. 6).

Immunization with SSVs and CSV confer protection in mice

The protective capacity provided by the CSV was determined by challenging mice immunized with 1×10^6 CFU of S2308. Four weeks post-challenge, the infection in each mouse was evaluated by determining the CFU in the spleen. The rL7/L12, rOMP22, rOMP25 and rOMP31 groups conferred significant degree of protection with 1.07-, 1.30-, 1.19- and 1.69-log units of protection compared with control mice receiving PBS, respectively. Mice immunized with adjuvant exhibited induced 0.09-log unit of protection compared with the PBS groups, but the result was not significant. Furthermore, immunization with CSV resulted in significantly higher degree of protection with 2.17- and 2.18-log units of protection compared with control mice receiving PBS and adjuvant respectively. By contrast, we immunized mice with the live-attenuated vaccine RB51. The RB51 group displayed the highest degree

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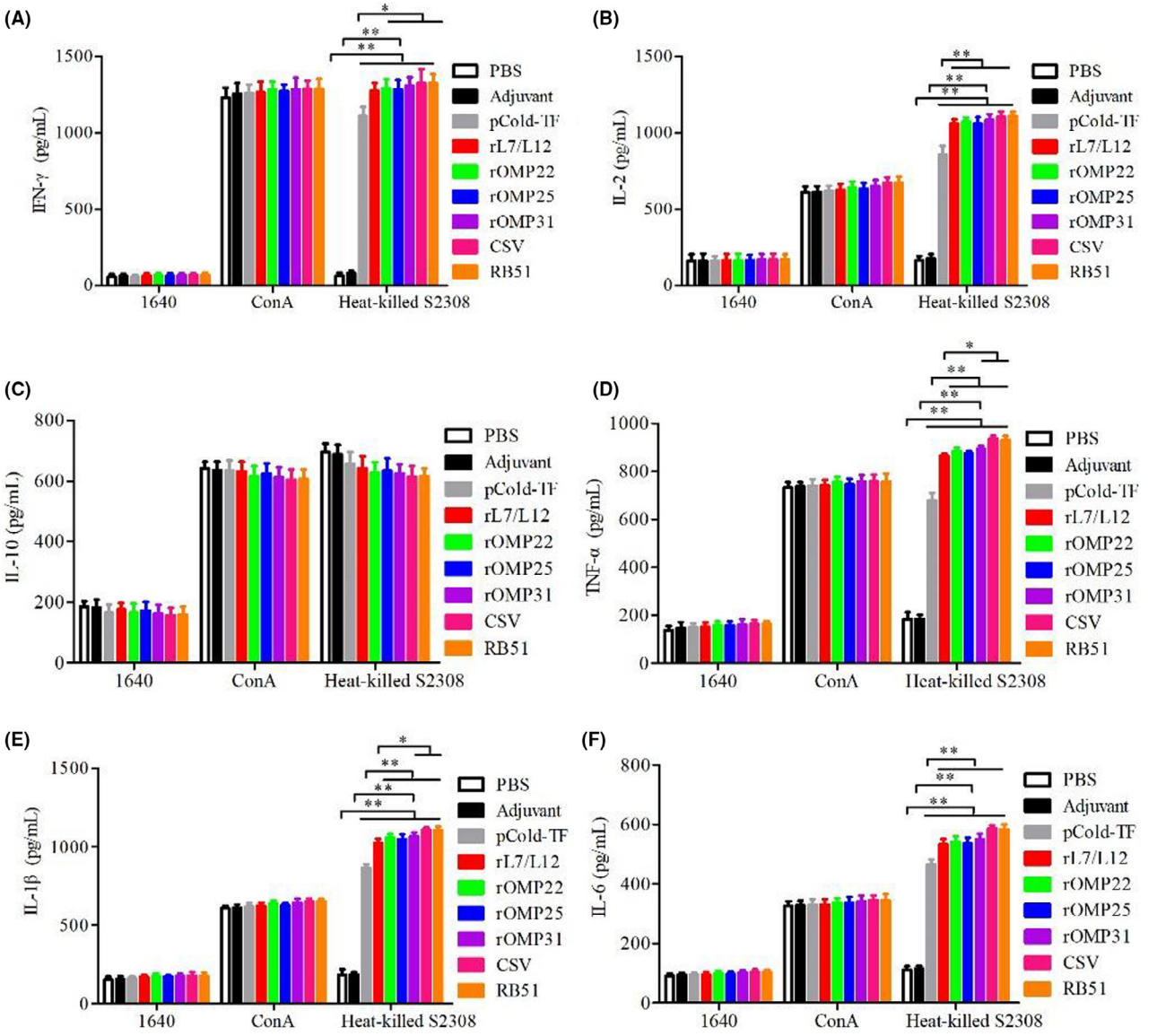

Fig. 4. Production of IFN-γ, IL-2, IL-10, TNF-α, IL-1β and IL-6 in stimulated splenocytes from BALB/c mice inoculated with PBS, adjuvant, pCold-TF, SSV, CSV and RB51. At week 8 after the last immunization, mice (n = 5 per group) were euthanized and spleen cells were isolated and stimulated with heat-killed S2308, ConA or RPMI 1640 medium as control. Splenocyte culture supernatants were harvested after 72 h stimulation. IFN-γ (A), IL-2 (B), IL-10 (C), TNF-α (D), IL-1β (E) and IL-6 (F) concentrations in the supernatant were measured by ELISA. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

of protection and induced 2.75-log unit of protection than the PBS group. The difference in the degree of protection amongst the SSV, RB51 and CSV groups was not significantly different (Table 1).

Discussion

Brucella spp. are intracellular pathogens that reside in host cells, including professional and non-professional phagocytes. The brucellae reside within macrophages where they replicate in a specialized compartment, namely, the *Brucella*-containing vacuole (BCV) (Celli and Gorvel, 2004). The membrane of the BCV contains endoplasmic reticulum (ER) proteins (Roy, 2002). Once inside the host macrophage, *Brucella* must fight against a variety of harmful conditions, including oxidative stress, decreased pH, scarcity of nutrients and pursuit of cellular and humoral immunity (Roop *et al*., 2009). However, the immune systems of mammals play an important role in resisting against pathogens. Furthermore, the activated macrophages, dendritic cells and cytokines are predominant in protection against *Brucella*.

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At present, vaccination is the effective way to prevent and control animal brucellosis. An ideal vaccine for brucellosis should be avirulent or attenuated for vaccinated animals to prevent *Brucella* infections, while not interfering with serological diagnoses (Schurig *et al*., 2002). In addition, it should be able to prevent abortion and virulence reversion and promote long periods of protection (Oliveira *et al*., 2010). However, current vaccines have major disadvantages that limit their application, although they can prevent infection in animals. Therefore, the development of a safe and effective vaccine is an arduous task.

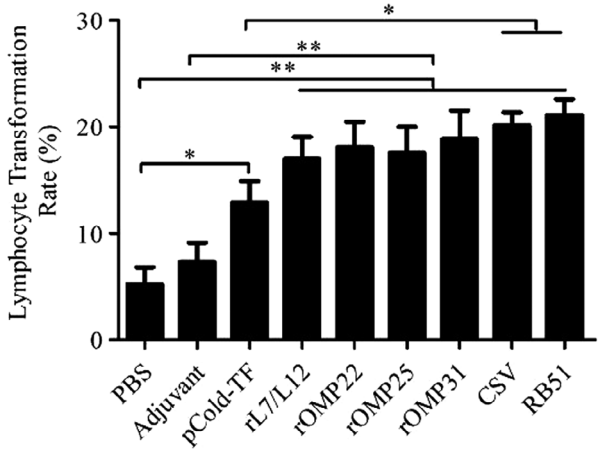

Fig. 5. Changes in T lymphocyte proliferation ratio in mice. Mice were immunized with purified rL7/L12, rOMP22, rOMP25 and rOMP31 for SSVs, CSV, PBS or adjuvant at weeks 0, 2, 4 and 6. As another positive control, mice were immunized with 1×10^6 CFU of RB51 at day 0. Peripheral blood samples were collected at week 8 after the last immunization. The ratio of T lymphocyte proliferation was tested. The values shown are means ± SD. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

Recently, some researchers have found that subunit vaccine was safe and non-infectious. Some studies have demonstrated that multivalent subunit vaccines prepared with multiple immunogens may provide better immune protection than monovalent vaccines (Hop *et al*., 2018; Gupta *et al*., 2019; Nazifi *et al*., 2019; Huy *et al*., 2020a, b; Rezaei *et al*., 2020; Shim *et al*., 2020). Therefore, the development of multivalent subunit vaccine may be an effective way to develop new generation vaccines.

Outer membrane proteins (OMPs) belong to surface antigens of *Brucella*. OMPs are considered as suitable candidates to produce recombinant proteins for prevention and diagnosis because of their protected sequences, antigenicity and high pathogenicity (Ducrotoy *et al*., 2016). OMPs played an important role in stimulating cellular and humoral immunity, which were recognized as protective and potentially immunogenic

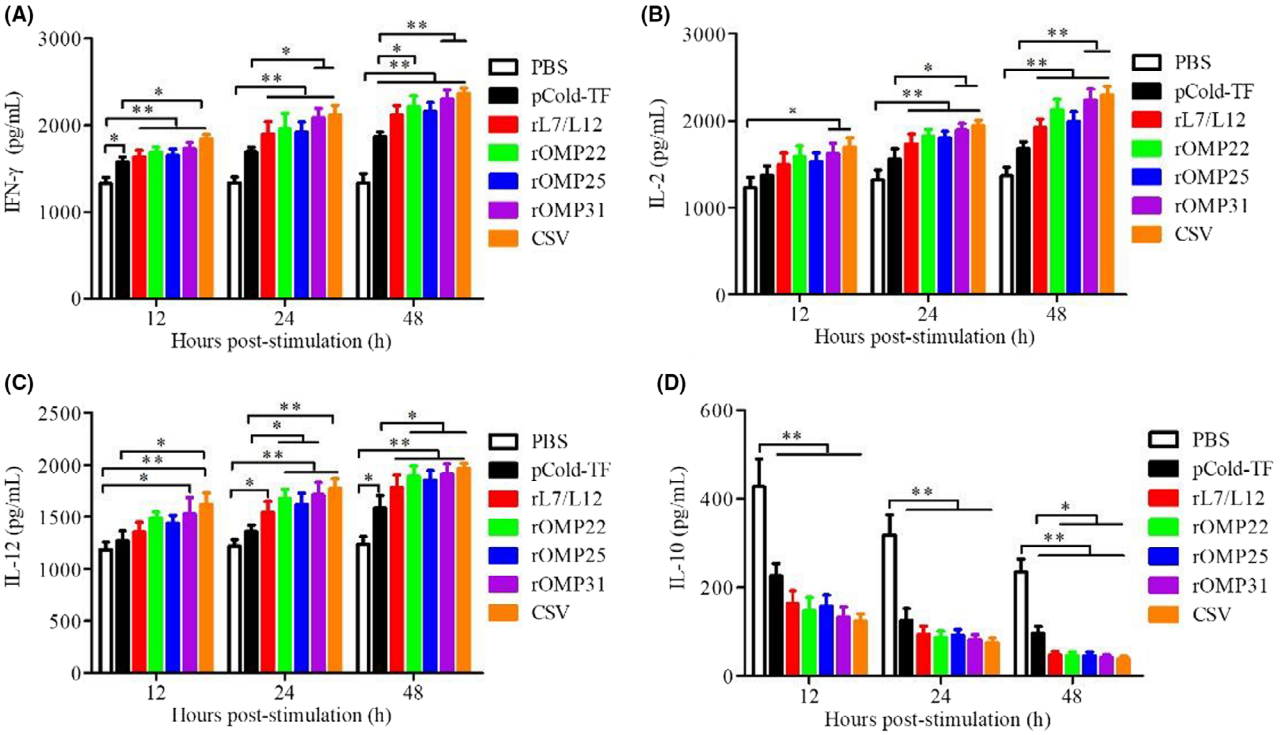

Fig. 6. Cytokine concentration in RAW 264.7 cell culture supernatants. After infection, 50 μl of cell culture supernatant was collected and analysed for cytokine production including IFN-γ (A), IL-2 (B), IL-12 (C) and IL-10 (D). The data are represented as the means ± SD of duplicate samples from at least two independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

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Table 1. Protection conferred by different vaccines against S2308 in mice.

Vaccine or control	Protection criteria		
	Log CFU of bacteria in spleens (mean ± SD)	Units of protection[b]	Significant[a]
PBS	5.92 ± 0.23	—	—
Adjuvant	5.83 ± 0.20	0.09	$P > 0.05$
pCold-TF	5.38 ± 0.38	0.54	$P < 0.05$
rL7/L12	4.85 ± 0.21	1.07	$P < 0.01$
rOMP22	4.62 ± 0.19	1.30	$P < 0.01$
rOMP25	4.73 ± 0.14	1.19	$P < 0.01$
rOMP31	4.23 ± 0.38	1.69	$P < 0.01$
CSV	3.65 ± 0.36	2.27	$P < 0.01$
RB51	3.17 ± 0.23	2.75	$P < 0.01$

a. Significant differences from PBS-immunized mice were estimated by Student's *t*-test.
b. Log units of protection = average of log CFU in spleens of control PBS-immunized mice minus the average of log CFU in spleens of vaccinated mice.

antigens (Sharghi *et al*., 2017). OMPs of *Brucella* were classified as 36- to 38-kDa OMPs (group 1 proteins), 31- to 34-kDa OMPs (group 2 proteins) and 25- to 27-kDa OMPs (group 3 proteins) based on their apparent molecular mass (Cloeckaert *et al*., 2002). Amongst these, OMP22 (also known as OMP3b), OMP25 (also known as OMP3a) and OMP31 belong to group 3 proteins. In addition, ribosomal protein L7/L12 functionally constitutes the 50S ribosome and plays an important role in controlling protein translational accuracy (Kirsebom and Isaksson, 1985). Based on previous reports, L7/L12, OMP22, OMP25 and OMP31 could stimulate strong immunity against *Brucella* infection (Goel and Bhatnagar, 2012; Zheng *et al*., 2015; Du *et al*., 2016; Minhas *et al*., 2021). Therefore, in this study, a combination of four recombinant proteins L7/L12, OMP22, OMP25 and OMP31 can activate strong immune responses against *Brucella* infection in RAW 264.7 cells and BALB/c mice.

Host immunity involves cellular immunity and humoral immunity during *Brucella* infection. The former is characterized by the production of IgG2a antibodies, whereas the latter is characterized by the production of IgG1 antibodies. IgG1 and IgG2a antibodies play an important role in the clearance of *Brucella* by macrophages (Perkins *et al*., 2010). Based on previous reports, the immune responses peaked after three independent immunizations of recombinant proteins (Hop *et al*., 2015). BALB/c mice were intraperitoneally (i.p.) injected with PBS, adjuvant, pCold-TF, SSVs or CSV to detect the effect of humoral immunity. Serum samples from mice were collected at 2, 4, 6, 8 and 10 weeks after the last immunization. Antibodies were detected in the sera of mice at 2 weeks after the last immunization, and IgG1 and IgG2a levels increased with time, and the IgG1 and IgG2a levels peaked at week 8 after the last vaccination (data not shown). IFN-γ can stimulate Th1 differentiation, which elicits the production of IgG2a from activated plasma cells (Motaharinia *et al*., 2013). IFN-γ is required for macrophage bactericidal activity, which activates macrophages to enhance killing and inhibit microbial replication (Dorneles *et al*., 2015). IFN-γ plays an important role in eradicating intracellular *Brucella* during the early stages of infection (Luo *et al*., 2006). In the present study, the production of IFN-γ was increased in CSV-immunized mice and CSV-infected RAW 264.7 cells compared with control. TNF-α and IL-6 are known as key effectors in mediating macrophages against *Brucella* infection. TNF-α is important for the influx of phagocytes to the site of infection and for macrophage activation, and it is critically involved in immune responses to intracellular pathogens (Zhan *et al*., 1996). IL-1β can induce the expression of several chemokines and adhesion molecules to enhance the phagocytic activity of neutrophils and monocytic cells (Hielpos *et al*., 2018). The present results showed that the production of proinflammatory cytokines TNF-α, IL-6, IL-2 and IL-1β was elevated in the CSV group. IL-12 promotes efficient immune responses against intracellular pathogens. During *Brucella* infection, IL-12 controls the intracellular growth of *Brucella* strains within macrophages (Macedo *et al*., 2008). We found that CSV-infected RAW 264.7 cells induced IL-12 production compared with the PBS group. By contrast, cytokine IL-10 can inhibit activity of macrophages and increase resistance of *Brucella* infection (Fernandes and Baldwin, 1995). The results in the present study showed that CSV-immunized mice and CSV-infected RAW 264.7 cells displayed decreased production of IL-10 compared with control. However, the concentration of IFN-γ was greater than that of IL-10 in the CSV group *in vitro* and *in vivo*. Furthermore, the levels of humoral immune response mediated by IgG antibodies in the serum are important for evaluating the immunogenicity of brucellosis. Elevated IgG1 and IgG2a antibody production in sera in SSV- and CSV-vaccinated mice was observed compared with PBS or adjuvant-immunized ones. These results indicated that SSVs and CSV induced strong cellular and humoral immune responses *in vivo* and *in vitro*.

Despite the absence of virulent for the host, the ideal vaccine must induce higher protection. Therefore, we detected the protection of the SSVs and CSV in BALB/c and found the superior protective effect conferred by SSVs and CSV. Immunization with SSVs or CSV conferred a significant level of protection compared with the PBS and adjuvant groups. Although the live-attenuated vaccine (RB51) conferred the highest protection against *Brucella* infection in this study, which was higher than mice immunized with SSVs or CSV, this difference was

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not significant. We also found that adjuvant could elicit protection against *Brucella* infection, but its protective effect was not significant.

In conclusion, our study clearly indicated that a combination of four different immunogenic antigens, namely, rL7/L12, rOMP22, rOMP25 and rOMP31, could significantly induce cell-mediated immunity response and humoral immunity response as compared with the PBS and adjuvant groups. In addition, CSV maintained a protective efficacy against *Brucella* infection similar to that of the RB51 vaccine strain. Therefore, CSV may be a suitable CSV candidate. However, this study was conducted in a murine model, which is not a natural host of *B. abortus*. Therefore, the efficacy and safety of this vaccine in livestock should be further investigated.

Experimental procedures

Ethical statement

The study was performed in strict accordance with the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Shihezi University (Permit No. SHZU-MO-0139). All animal handling procedures were approved by the Committee on the Ethics of Animal Experiments of Shihezi University (Authorization Number: 2021-2). The animal operation procedures were implemented on the basis of the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). All efforts were made to minimize suffering during animal handling and experimentation. The mice were kept in biosafety level 3 (BSL-3) animal facilities.

Bacterial strains and growth condition

The virulent, wild-type *B. abortus* 2308 strain (S2308) and *B. abortus* RB51 vaccine strain were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). All *Brucella* strains were cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB, Difco, MI, USA) at 37°C in 5% CO_2. All experiments related to live S2308 and RB51 were performed in a biosafety level 3 (BSL-3) laboratory. *Escherichia coli* (*E. coli*) strains DH5α and BL21 were purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* cultures were grown at 37°C in a Luria–Bertani (LB) medium (Difco, MI, USA) supplemented with 100 μg ml^{-1} of ampicillin (Sigma, Missouri, USA) for the expression of recombinant proteins.

Vector, cell culture and mice

The pCold™ Trigger factor (TF) vector (pCold-TF) was purchased from Takara (Shiga, Japan). The RAW 264.7 (ATCC) murine macrophage line (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM) basic (Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD, USA) without antibiotics and incubated at 37°C in 5% CO_2 (v/v) atmosphere. Female 6-week-old BALB/c mice were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). The animals were provided with humane care and healthful conditions during their stay in the facility. All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

Cloning, expression and purification of recombinant proteins

Expression of recombinant plasmids and purified recombinant proteins was performed as described previously (Im *et al*., 2018; Huy *et al*., 2020a,b). In brief, the open reading frames (ORFs) of the four *B. abortus* genes, namely, *L7/L12* (BAB1_1265, 375bp), *omp22* (BAB1_1302, 639bp), *omp25* (BAB1_0722, 642bp) and *omp31* (BAB1_1639, 786bp), were amplified by PCR from the S2308 genome using their respective primer pairs (Table 2). The amplified DNA fragments were cloned into the pCold-TF vector to generate recombinant plasmids, namely, pCold-L7/L12, pCold-Omp22, pCold-Omp25 and pCold-Omp31. Then, these recombinant plasmids were transformed into *E. coli* BL21 cells. Afterward, the final clones obtained on LB agar plates supplemented with ampicillin (100 μg ml^{-1}) were inoculated into LB broth supplemented with ampicillin overnight. This overnight culture was used for further inoculation in fresh LB broth induced with 1 mM of IPTG at 0.6 OD. The induced and uninduced fragments were collected at 6 h after the induction. 12% SDS-PAGE analysis of the collected fragments was performed using a 5× sample lysis buffer. Five millilitres of induced bacterial pellet was dissolved in lysis buffer to verify whether the protein was soluble or could form inclusion bodies (50 mM of NaH_2PO_4, 300 mM of NaCl, 10 mM of imidazole and 1 mg ml^{-1} of lysozyme), incubated on ice and then sonicated (Vibra cell, Sonics, USA) at 40 W amplitude for 10 min and 8 s of pulse. The sonicated suspension was then centrifuged at 9000 *g* for 15 min at 4°C. Pellet and supernatant were collected and checked for the expressed recombinant protein by SDS-PAGE analysis for insoluble and soluble protein respectively.

Recombinant proteins, namely, rL7/L12, rOMP22, rOMP25 and rOMP31, were expressed in *E. coli* BL21 and were purified by chromatography through Ni-NTA agarose (Qiagen) in accordance with the manufacturer's instructions. As described previously, these recombinant

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Table 2. Primers used in this study.

Primer		5′-3′ sequence	
L7/L12-F	Forward	*TCTAGA*ATGGCTGATCTCGCAAAGATC (*Xba* I)	Amplification L7/L12
L7/L12-R	Reverse	*CTGCAG*TTACTTGAGTTCAACCTTGGC (*Pst* I)	
OMP22-F	Forward	*GGATCC*ATGTTCAAGCGTTCTATCACC (*Bam*H I)	Amplification OMP22
OMP22-R	Reverse	*AAGCTT*CTAGAATTTGTAGTTCAGGCC (*Hin*d III)	
OMP25-F	Forward	*GGATCC*ATGCGCACTCTTAAGTCTCTC (*Bam*H I)	Amplification OMP25
OMP25-R	Reverse	*CTGCAG*TTAGAACTTGTAGCCGATGCC (*Pst* I)	
OMP31-F	Forward	*GGATCC*ATGTTTAGCTTAAAAGGGACTGTT (*Bam*H I)	Amplification OMP31
OMP31-R	Reverse	*CTGCAG*TTAGAACTTGTAGTTCAGACCGAG (*Pst* I)	

proteins were purified under denaturing conditions because they were forming inclusion bodies (Pathak *et al*., 2018). In brief, 50 ml of induced bacterial culture was pelleted and washed with PBS two times. The washed pellet was dissolved in 8 ml of lysis buffer [8 M of urea, 100 mM of NaH_2PO_4, 10 mM of Tris-Cl, 20 mM of β-mercaptoethanol, 1% Triton X-100, 1 mM of phenylmethylsulfonyl fluoride (PMSF), pH 8.0], sonicated for 10 min and incubated for 1 h with continuous stirring at room temperature and 180 rpm. After 1 h incubation, the lysed cell suspension was centrifuged at 9000 *g* for 30 min at room temperature and supernatant was collected and mixed with Ni-NTA agarose (Qiagen) in 1:3 ratio (Super-flow Ni-NTA slurry: Lysate) for 1 h to allow the rL7/L12, rOMP22, rOMP25 and rOMP31 6×-His tagged protein to bind with Ni^{2+} in the super-flow slurry. The agarose super-flow Ni-NTA slurry-lysate mix was then packed in a 15 ml column, and then flow-thru was collected. Furthermore, the column was washed with 20 ml of wash buffer (8 M of urea, 100 mM of NaH_2PO_4 10 mM of Tris-Cl, 1% Triton X-100, 10% glycerol, pH 6.3) to remove the unbound or weakly bound non-specific proteins. Then, the rL7/L12, rOMP22, rOMP25 and rOMP31 proteins were eluted from the column using elution buffer (8 M of urea, 100 mM of NaH_2PO_4, 10 mM of Tris-Cl, pH 4.5) in 1 ml of 5 fractions and further analysed on 12% SDS-PAGE. Refolding or dialysis of the purified porin protein was performed against a decreasing urea gradient (8, 6, 4, 2 and 1 M) and finally against PBS. The expression and immunoreactivity of these recombinant proteins were analysed by 12% SDS-PAGE and Western blot as described previously (Li *et al*., 2018a,b; Zhu *et al*., 2020).

Immunization of mice

Mice immunization was conducted as previously described (Hop *et al*., 2018) with some modifications to evaluate the immunogenicity of these expression proteins. In brief, a total of 45 six-week-old female pathogen-free (SPF) BALB/c mice were randomly distributed into eight groups of five mice each and reared under identical environmental conditions. Each mouse was i.p. injected with a mixture of incomplete Freund's adjuvant (IFA, Sigma-Aldrich, Missouri, USA) and 100 μg of purified pCold-TF, rL7/L12, rOMP22, rOMP25 or rOMP31 in a total volume of 200 μl at weeks 0, 2, 4 and 6 for the SSV groups, or 100 μg of purified rL7/L12, rOMP22, rOMP25 and rOMP31 at a ratio of 1:1:1:1 in a total volume of 200 μl at weeks 0, 2, 4 and 6 for the CSV group. The reference vaccine group was i.p. inoculated with 200 μl of PBS containing 1×10^6 CFU of RB51. The negative control group was i.p. injected with 200 μl of PBS or adjuvant. Mice immunized with different vaccines were performed in a BSL-3 laboratory. All the performed procedures were approved by the Committee on the Ethics of Animal Experiments of Shihezi University (Authorization Number: 2021-2).

Measurement of antibody levels from serum samples

The IgG1 and IgG2a antibody levels were detected using indirect enzyme-linked immunosorbent assay (iELIAS) as previously described to evaluate humoral immunity induced by SSVs or CSV (Huy *et al*., 2020a,b). Serum samples were obtained from peripheral blood (tail vein) of immunized BALB/c mice at week 8 after the last immunization (Zhang *et al*., 2013). The IgG1 and IgG2a antibodies in serum samples obtained from immunized mice were determined by iELISA as previously described (Li *et al*., 2017). The high binding 96-Well Single-Break Strip Plates were coated with fully inactivated bacteria at 100 μl overnight at 4°C. Horseradish peroxidase-conjugated goat anti-mouse IgG1 and IgG2a antibodies were used to measure. The absorbance at 450 nm (OD_{450}) of each well was detected at 30 min after substrate addition. All assays were performed in triplicate.

Measurement of cytokine concentrations from serum samples and splenocytes

The levels of IFN-γ, IL-2, IL-10, TNF-α, IL-1β and IL-6 in serum samples were determined using mouse cytokine ELISA kits (ebioscience, San Diego, CA, USA) in

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accordance with the manufacturer's instructions. All assays were performed in triplicate.

At week 8 after the last immunization, BALB/c mice ($n = 5$ per group) were euthanized and their spleens were removed aseptically. Single-cell suspensions were obtained from the spleens by homogenization. The cells were suspended in complete RPMI 1640 medium (Gibco Life Technologies, Rockville, MD, USA) supplemented with 2 mM of L-glutamine (Solarbio Science and Technology, Beijing, China) and 10% (v/v) FBS. Erythrocytes were eliminated with ACK lysis solution (150 mM of NH_4Cl, 1 mM of Na_2-EDTA, pH 7.3). Splenocytes (5×10^5 cells/well) were cultured in 96-well plates; the cultures were stimulated by adding 40 μg of heat-killed S2308 lysate/well, 1 μg of Concanavalin A (ConA) (positive control) or medium alone (negative control). The cells were incubated at 37°C with 5% CO_2 (v/v) for 72 h. Subsequently, the clear culture supernatants were collected and stored at −20°C until tested. IFN-γ, IL-2, IL-10, TNF-α, IL-1β and IL-6 levels in the supernatants were measured using mouse cytokine ELISA kits (eBioscience) in accordance with the manufacturer's instructions.

Methyl thiazol tetrazolium (MTT) assay

Fresh anticoagulated peripheral blood samples from five mice (1.0 ml per mice) were collected in each group at week 8 after the last immunization to evaluate the proliferation of peripheral blood lymphocytes (PBL). The proliferation of PBL was detected by the MTT method as previously described (Mwanza *et al*., 2009; Zhu *et al*., 2020). The optical density (OD) at 490 nm was measured using a spectrophotometer. Lymphocyte transformation rate (LTR) = (mean of Con A stimulation group − mean of non-Con A stimulation group)/mean of non-Con A stimulation group.

Measurement of cytokine concentrations in RAW 264.7 cells

RAW 264.7 cells of 2×10^5 cells/well were cultured in 96-well plates at 37°C under 5% CO_2 overnight. Cells were pretreated with SSVs or CSV for 4 h. The control group was pretreated with PBS. The cells were washed with PBS, incubated in fresh medium (DMEM with 10% heat-inactivated FBS) and then infected with S2308 at a multiplicity of infection (MOI) of 100. Culture plates were centrifuged at 350 *g* for 5 min at room temperature and incubated at 37°C for 45 min. After washing two times with medium without antibiotics, the infected cells were incubated for 60 min in the presence of 50 μg ml^{-1} of gentamicin to kill extracellular bacteria. Then, the cultures were placed in fresh DMEM containing 25 μg ml^{-1} of gentamicin (defined as time zero), and treated with pCold-TF, SSVs or CSV for 12, 24 and 48 h. At 12, 24 and 48 h post-treatment, 50 μl of cell culture supernatant from each well was collected to evaluate the levels of IFN-γ, IL-2, IL-12p70 and IL-10 using mouse cytokine ELISA kits (eBioscience) in accordance with the manufacturer's instructions. All assays were performed in triplicate.

Protection induced by SSVs and CSV

Protective efficacy of SSVs and CSV was performed as previously described (Golshani *et al*., 2018a,b; Li *et al*., 2018a,b) with some modifications. In brief, mice were immunized using the above-mentioned methods. At week 8 after the last immunization, mice were i.p. challenged with approximately 1×10^6 CFU of S2308 virulent strain in 200 μl of PBS. The mice were euthanized at week 4 post-challenge, and their spleens were removed aseptically. Each spleen was homogenized in 1 ml of PBS containing 0.1% (v/v) Triton X-100, ten-fold serially diluted and then plated on TSA plates. Plates were incubated at 37°C, and the number of CFU per spleen was counted after 3 days. The unit of protection was calculated as the mean $\log_{10}$ CFU of the PBS group minus $\log_{10}$ CFU of the experimental group. Animal challenges with S2308 were performed in the BSL-3 laboratory. All the performed procedures were approved by the Committee on the Ethics of Animal Experiments of Shihezi University (Authorization Number: 2021-2).

Statistical analysis

Antibody response was expressed as the mean Log titre ± the standard deviation (SD). Cytokine production was expressed as the mean cytokine concentration ± SD. The lymphocyte proliferation rate was expressed as the mean percentages for analysis ± SD. The protective efficiency at different time points was expressed as the mean Log CFU ± SD. Statistical significance (*P* value) was calculated with Student's *t*-test using Statistical Package for Social Science (SPSS) software version 19.0 (MS, Chicago, IL, USA). *P* values of < 0.05 were considered statistically significant.

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Author contributions

ZL and HZ designed the experiments. ZL, SW, GY, CZ, LX, JZ, YC, JH and HZ performed the experiments and analysed the data. ZL and HZ contributed reagents/materials/analysis tools. ZL, SW and HZ wrote and revised the paper.

Conflict of interest

The authors have declared that there are no competing interests.

Data availability statement

All data generated or analysed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

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