Veterinary Microbiology 229 (2019) 39–47

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Newcastle disease virus-like particles containing the *Brucella* BCSP31 protein induce dendritic cell activation and protect mice against virulent *Brucella* challenge

Xiaohong Xu[a,1], Zhuang Ding[a,1], Jindou Li[a], Jiaming Liang[b], Zhaoyang Bu[b], Jiaxin Ding[a], Yanling Yang[c], Xulong Lang[b], Xinglong Wang[b], Renfu Yin[a,*], Jing Qian[d,*]

[a] *Laboratory of Infectious Diseases, College of Veterinary Medicine, Key Laboratory of Zoonosis Research, Ministry of Education, Jilin University, Changchun 130062, China*

[b] *Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary Medicine, Academy of Military Medical Sciences, Changchun 130122, China*

[c] *Institute of Special Wild Animal & Plant Science, Chinese Academy of Agricultural Sciences, Changchun 130122, China*

[d] *Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences / Key Laboratory for Veterinary Bio-Product Engineering, Ministry of Agriculture, Nanjing 210014, China*

ARTICLE INFO

Keywords:
Newcastle disease virus-like particles
Brucella BCSP31 protein
Dendritic cell maturation
Protective efficacy
Vaccine candidate

ABSTRACT

Brucellosis is a widespread zoonosis that poses a substantial threat to human and animal public health due to the absence of a sufficiently safe and efficient vaccine. Virus-like particles (VLPs) have been developed as novel vaccine candidates and suitable carrier platforms for the delivery of exogenous proteins. Herein, we constructed chimeric virus-like particles (cVLPs) assembled by a Newcastle disease virus (NDV) M protein and glycosyl-phosphatidylinositol-anchored *Brucella* BCSP31 protein (GPI-BCSP31). cVLPs-GPI-BCSP31 were highly efficient in murine dendritic cell (DC) activation, both *in vitro* and *in vivo*. Moreover, they elicited strong specific humoural immune responses detected through ELISA assay with inactivated *Brucella* and recombinant BCSP31 protein and by elevated cellular immune responses indicated by intracellular IFN-γ and IL-4 levels in CD3 + CD4 + T and CD3 + CD8 + T cells. Importantly, cVLPs-GPI-BCSP31 conferred protection against virulent *Brucella melitensis* strain 16 M challenge, comparable to the efficacy of *Brucella* vaccine strain M5. In summary, this study provides a new strategy for the development of a safe and effective vaccine candidate against virulent *Brucella* and further extends the application of NDV VLP-based vaccine platforms.

1. Introduction

Brucellosis, caused by the gram-negative facultative intracellular pathogen *Brucella*, is a worldwide zoonotic disease, resulting in infertility and paralysis in both humans and livestock (Hanson, 2006; Seleem et al., 2010). There are currently ten recognized species of *Brucella*, all of which are named based on their natural host (Hanson, 2006; Mp et al., 2014; Wattam et al., 2009) and four of which are pathogenic to humans (virulence in descending order): *Brucella melitensis, Brucella suis, Brucella abortus*, and *Brucella canis*. *Brucella* species are difficult for the body to clear due to their ability to infect and replicate within antigen presenting cells (APC), such as macrophages and dendritic cells (DC), the very cells that are pivotal for appropriate adaptive immune responses (Archambaud et al., 2010). Therefore, swift and robust APC activation is crucial in the course of *Brucella* clearance.

Prevention strategies for brucellosis primarily rely on the use of attenuated live vaccines, including *B. melitensis* strain 5 (M5), *B. melitensis* strain Rev. 1 (Rev.1), *B. abortus* strain 19 (S19), *B. abortus* strain RB51 (RB51), and *B. suis* strain 2 (S2) (Avila-Calderón et al., 2013; Schurig et al., 2002). Each of these were designated for a very specific animal species and have a relatively limited protection efficacy, ranging from 65% to 80% (Avila-Calderón et al., 2013; Blasco et al., 1993; Crawford et al., 1991; Olsen and Johnson, 2012; Verger et al., 1995). Another factor that may impede their application is that live vaccine strains are pathogenic to young and immunocompromised individuals. Furthermore, their inherent risk for replication in the host makes it impossible for them to eradicate the pathogen from the herd. Therefore, a safer and more efficient vaccine candidate has long been needed. The

* Corresponding authors.
E-mail addresses: yin@jlu.edu.cn (R. Yin), qjysb0110@126.com (J. Qian).
[1] Contribute equally to the manuscript.

https://doi.org/10.1016/j.vetmic.2018.12.007
Received 28 August 2018; Received in revised form 15 November 2018; Accepted 10 December 2018

BCSP31 protein, a 31 kDa bacterial cell surface protein, was proven to be an important protective antigen protein of *Brucella* and is highly conserved across species (Bricker et al., 1988; Li et al., 2014). It has proven its immunogenicity through a recombinant protein, a DNA vaccine, and a live-vector in mice and exhibited protective efficacy in several studies (Kim et al., 2016; Li et al., 2014; Yu et al., 2007).

Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, potentially yielding safer and less expensive vaccine candidates (Roldão et al., 2010). Taking advantage of their suitable size and well-preserved antigen spatial structure, VLPs can be efficiently taken up and processed by APCs to form MHC-polypeptide complexes (Manolova et al., 2010). We previously demonstrated that Newcastle disease virus-like particles (NDV VLPs) can promptly induce DC maturation, leading to upregulation of MHC-II, co-stimulatory molecules, and proinflammatory cytokines (IFN-γ and IL-12) and their migration to the T cell area of lymph nodes, which subsequently differentiate into Th1, Th2, and Tc cells (Qian et al., 2017b). Their DC activating ability makes them a good adjuvant when was used as a vaccine vector transporting foreign antigens. However, application of BCSP31 protein on VLPs has not been reported.

NDV VLPs are proven competent carrier platforms for exogenous proteins, such as F and G protein of the respiratory syncytial virus (RSV) and G protein of Nipah virus (Mcginnes et al., 2011, 2010). However, current NDV VLP modifying strategies are limited to the replacement of the ectodomain of NDV F or HN protein, in which only type I and II transmembrane proteins can integrate into NDV VLPs. Glycophosphatidylinositol (GPI) is a cell surface protein anchored to the eukaryotic cell membrane that envelopes VLPs without crossing the phospholipid bilayer (Patel et al., 2015; Schroeder et al., 1994). Patel et al. generated GPI-fused immune-enhancing adjuvants, such as GM-CSF, ICAM-1, and IL-12, which maintained a prolonged half-life period and could be anchored to the surface of avian influenza virus-like particles (AIV VLPs). The results of mouse immunization showed that GPI-GMCSF-VLP induced stronger antibody responses and better protection against a heterologous influenza virus challenge than did unmodified VLPs (Patel et al., 2015).

In this study, the BCSP31 gene was fused to a GPI anchor sequence, and the GPI-BCSP31 protein was expressed using an insect baculovirus expression system and subsequently anchored to NDV VLPs through co-infection with rBV-M and rBV-GPI-BCSP31. The resultant chimeric virus-like particles (cVLPs) efficiently induced DC maturation both *in vitro* and *in vivo*. They also provided competent immunogenicity through strong humoural and cellular immunity, and full protection against *B. melitensis* 16 M challenge *in vivo*. In summary, we developed a safe and efficient vaccine candidate against a virulent *Brucella* strain using a GPI modifying strategy, which not only provides a suitable vaccine candidate for Brucellosis elimination but also extends the utilization of NDV VLP-based vaccine platforms.

2. Materials and methods

2.1. Virus, bacterial strains, plasmids, cell lines, and animals

A recombinant baculovirus expressing the NDV M gene (rBV-M) was previously constructed in our lab (Qian et al., 2017b). *Brucella* vaccine M5 and virulent *B. melitensis* 16 M strains were grown on tryptic soy broth (TSB, Sigma, St. Louis, MO, USA) agar at 37 °C. All live *Brucella* manipulations were performed in a BSL-3 facility. The Bac-to-Bac insect-baculovirus expression system (Life technologies corporation, Gaithersburg, MD, USA), consisting of pFastBac1 transfer vector, *E. coli* DH10Bac competent cells, and Sf9 insect cells, was stored in our lab. BALB/c mice (female, eight weeks old) were purchased from the Changchun Institute of Biological Products Co., Ltd, China (Approval numbers SYXK-JI-2016-0001). All animal experiments were performed according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University and the Institute of Veterinary Medicine of the Military Academy of Sciences. Challenged mice were housed in an animal biosafety level 3 (ABSL-3) facility.

2.2. Construction of recombinant baculovirus and expression of the GPI-BCSP31 protein

The artificially designed and synthesized fusion sequence includes a Kozak sequence, a mimetin secretion signal (from Baculovirus expression vector pFastBac1-HM, GenBank: AY598466), a His-tag, multiple cloning sites (containing *Sal* I, *Xba* I, *Pst* I, *Xho* I and *Kpn* I restriction enzyme sites), and a GPI-signal sequence from CD55 (mouse CD55 sequence, GenBank: NM_010016). Subsequently, the fusion sequence was introduced into the pFastBac1 transfer vector by restriction endonucleases *Eco*R I and *Hin*d III (New England Biolabs, Ipswich, MA, USA) to construct the pFastBac1-GPI chimeric vector. The BCSP31 gene was derived from *B. melitensis* 16 M (provided by Dr. Wang Xinglong from the Military Academy of Sciences), and the extracellular domain sequence was determined by transmembrane region analysis software (TMHMM Server v. 2.0). Primers were designed with the following sequences: BCSP31-F, 5′-GTCGACATGCGTATCGGCACTGG-3′ containing *Sal* I restriction site (underlined) at 5′ ends and BCSP31-R, 5′-GGTACCTTTCAGCACGCCCGC -3′ containing *Kpn* I restriction site (underlined) at 5′ ends. PCR conditions were as follows: 95 °C 5 min; 94 °C 30 s, 60 °C 30 s, and 72 °C 1 min for 30 cycles; 72 °C 10 min.

The BCSP31 gene was inserted into the pFasBac1-GPI vector using the *Sal* I and *Kpn* I sites. Subsequently, the recombinant baculovirus (rBV) containing chimeric the GPI-BCSP31 sequence was generated using the Bac-to-Bac insect-baculovirus expression system. rBV-GPI-BCSP31 was inoculated with suspended Sf9 cells (MOI = 3) and cultured at 27 °C for three days. Cells were collected by centrifugation at 2000 × g and resuspended in equilibration buffer. After ultrasonic disruption (200 W, broken 10 s, interval 10 s, repeated five times), the suspension was centrifuged at 8000 × g for 30 min at 4 °C to collect the supernatant. The GPI-BCSP31 protein was purified by affinity chromatography (HisTrap™ HP Columns, GE Healthcare), quantified by a Pierce BCA Protein Assay Kit (Pierce Thermo Scientific, Rockford, IL, USA), and examined by SDS-PAGE using 12% polyacrylamide gels. For western blot analysis, proteins in polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes and then incubated with 1:500 dilution of primary mouse anti-BCSP31 polyclonal antibody (provided by Dr. Jing Qian from the Jiangsu Academy of Agricultural Sciences) followed by 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (H + L) secondary antibody (EarthOx, San Francisco, CA, USA).

2.3. Preparation and characterization of VLPs

Control VLPs were produced by infecting rBV-M at an MOI = 5 for 72 h at 27 °C, as described previously (Qian et al., 2017b). To obtain virus-like particles anchored with GPI-BCSP31 protein, rBV-GPI-BCSP31 and rBV-M were co-infected into Sf9 cells at an MOI ratio of 3:2 at 27 °C for 72 h. Titres of the rBVs were determined using the BacPAK™ Baculovirus Rapid Titer Kit (Clontech, Palo Alto, CA, USA), and the infected supernatant was then collected. Before purification, the supernatant was treated with 0.1% beta-propiolactone (BPL) (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 3 h on ice, followed by incubation for 2 h at 37 °C to remove BPL. Inactivation of BV was confirmed by incubation with Sf9 for three serial generations with no signs of pathology. The product was subjected to centrifugation at 8000 × g for 30 min and pelleted at 100,000 × g at 4 °C for 3 h. Pellets were collected and resuspended in phosphate buffered saline (PBS, pH 7.2) and went through a 20-40-60% (w/v) sucrose gradient, as previously described (Qian et al., 2017b). The cVLPs-GPI-BCSP31 of the intermediate layer were diluted with PBS and centrifuged at 100,000 ×

g at 4 °C for 1 h to remove sucrose. Finally, purified VLPs were re-suspended in TNE buffer (0.150 M NaCl, 0.025 M Tris-Cl, 0.005 M EDTA, pH 7.4) and stored at −80 °C until use. The combination of GPI-BCSP31 and NDV VLP (M protein based) was confirmed by western blot using anti-BCSP31 and anti-M antibodies, respectively. To produce anti-M antibodies, the M gene sequence of the NDV NA-1 strain was cloned into the pET-28a vector containing a His-tag. The antigen was then expressed through an *E.coli* expression system, purified with immobilized affinity chromatography, and administered to rabbits with the assistance Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Serum was collected to produce anti-M polyclonal antibody, which was further purified through protein A/G affinity chromatography. Its cross-reaction with other NDV structural proteins, baculovirus, or insect protein was quite low. Production efficiency of total cVLPs-GPI-BCSP31 was quantified through BCA assay. To access the purity of 10 μg cVLPs-GPI-BCSP31, it was resolved onto 12% SDS-PAGE, as well as different concentrations of BSA. After stained with coomassie blue and subjected to greyscale analyse using ImageJ software, a standard curve based on the densitometry of the BSA was obtained, and the concentration of NDV M and GPI-BCSP31 proteins were hence determined.

The morphology and structure of the purified control VLPs and cVLPs-GPI-BCSP31 were observed by immunoelectron microscopy (IEM). Briefly, VLPs were adsorbed onto freshly discharged 400 mesh carbon parlodion-coated copper grids for 15 min and washed twice with PBS. The grids were incubated with a 1:50 dilution of mouse anti-BCSP31 polyclonal antibody at room temperature for 2 h, followed by a 1:100 dilution of goat anti-mouse IgG (whole)-gold conjugate (10 nm) (Sigma, St Louis, MO, USA). Finally, grids were negatively stained with 1% phosphotungstic acid and dried by aspiration. VLPs were visualized on a Hitachi H-7650 transmission electron microscope (Hitachi Ltd, Tokyo, Japan) operating at 80 kV.

2.4. Detection of DC activation in vitro and in vivo

Murine bone marrow cells of mouse femur and tibia were collected and cultured in appropriate complete medium, containing 1640 medium, 10% FBS, 1% penicillin and streptomycin, final concentration 10 ng/ml rmGM-CSF, and 10 ng/ml rmIL-4, for six days, as described by Qian et al (Qian et al., 2017b). Cells were plated at 5×10^6 cells/well into 6-well plates and treated with control VLPs (5 μg of total protein in 10 μL), cVLPs-GPI-BCSP31 (5 μg of total protein in 10 μL, including 0.85 μg of BCSP31 protein), soluble recombinant BCSP31 protein (rBCSP31, 0.85 μg of total protein in 10 μL, provided by Dr. Jing Qian), or PBS (10 μL) for four or 24 h under 5% CO_2 at 37 °C. After treatment, DCs were incubated with fluorescent antibodies or isotype controls (eBioscience, San Diego, CA, USA): PE-conjugated anti-mouse MHC II and CD86, Mean fluorescence intensity (MFI) was detected by flow cytometry to evaluate mature surface markers. The BCSP31 dose in cVLPs-GPI-BCSP31 was quantified by western blot compared with that of the equivalent dose of soluble rBCSP31 protein.

To estimate the phagocytic ability of DCs, 1×10^6 stimulated DCs were incubated with 1 mg/mL FITC-Dextran (MW 40000, Sigma-Aldrich, St. Louis, MO, USA) at 37 °C or at 4 °C (negative control) for 4 h and 24 h, as previously described (Qian et al., 2017b). MFI was measured by flow cytometry, and DC phagocytic capacity was evaluated by MFI difference (ΔMFI) according to the following formula: ΔMFI = MFI (37 °C treatment) – MFI (4 °C treatment). In addition, to assess their allogeneic stimulatory activity, mixed leukocyte reaction (MLR) was adopted, as previously described. Briefly, 5×10^5 treated DCs were co-cultured with 1×10^7 autologous naïve T cells from spleen (DC: T = 1:20) in 96-well plates at 37 °C for 48 h, followed by subsequent incubation with 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) for 4 h at 37 °C. The OD_{450} values of each group were measured to evaluate the activation ability of T cells according to the following formula: stimulation index (SI) = (ODsample well-ODblank well) / (ODnegative well-ODblank well).

To mimic *in vivo* DC activation as accurately as possible, BALB/c mice (female, eight weeks old) were randomly divided into four groups (n = 6) and immunized with control VLPs (5 μg of total protein in 25 μL), cVLPs-GPI-BCSP31 (5 μg of total protein in 25 μL, including 0.85 μg of BCSP31 protein), soluble BCSP31 protein (0.85 μg of total protein in 25 μL), or PBS (25 μL) by footpad intramuscular injection. The ipsilateral draining lymph nodes and spleens were isolated at 4, 24, 48 and 72 h after the injection, and 2×10^6 suspension cells were incubated with PerCP-Cy5.5 anti-mouse CD3e antibody, FITC anti-mouse CD11c antibody, and PE anti-mouse MHC II antibody to be used for flow analysis. Data analysis was performed using FlowJo software (Version 10.0.7).

2.5. Animal immunization

BALB/c mice (female, eight weeks old) were randomly divided into seven groups (n = 6) and immunized twice with PBS (100 μL/mouse), control VLPs (5 μg of total protein each mouse in 100 μL), cVLPs-GPI-BCSP31 (1, 5, and 10 μg of total protein each mouse in 100 μL containing 0.17, 0.85, and 1.7 μg of BCSP31 protein, respectively), soluble rBCSP31 protein (0.85 μg/mouse in 100 μL), or *Brucella* M5 strain (M5, 1×10^7 CFU/mouse in 100 μL). The initial injection was considered day 0, the boost (the M5 strain group was not subjected to boost) occurred at day 14, and serum was collected through the tail vein each week.

2.6. Antibody response assessment

The specific IgG antibody titre was determined by indirect ELISA, as previously described (Qian et al., 2017a). The 96-well plates (Costar, Corning, NY, USA) were coated with diluted BCSP31 protein (pH 9.6, 0.05 M carbonate buffer 100 μL/well of 0.5 μg) and diluted inactivated *Brucella* M5 strain (pH 9.6, 0.05 M carbonate buffer 100 μL/well of bacteria at a concentration of 10^7 CFU) at 4 °C overnight. Following washing with PBST (PBS containing 0.5% Tween-20), wells were blocked using 1% bovine serum albumin (BSA) for 1 h at 37 °C. Diluted serum (1:1000) was used as the primary antibody and incubated at 37 °C for 1 h followed by washing three times with PBST. Subsequently, goat anti-mouse IgG-HRP (1:5000) was added as the secondary antibody and incubated at 37 °C for 1 h. Next, 100 μL of TMB substrate solution (Tiangen Biotech Co. Ltd., Beijing, China) was added and incubated at 37 °C for 15 min in the dark, followed by addition of 50 μL of stop solution (2 M H_2SO_4) to measure OD_{450} values after 10 min.

2.7. Analysis of T lymphocyte differentiation

Four weeks after boost (at week 6), spleens were collected aseptically to prepare a single-cell lymphocyte suspension, as previously described (Qian et al., 2017a). Briefly, the spleen was soaked in 4 mL of lymphocyte separation liquid (Dakewe Biotech Co., Shenzhen, China) and milled in a 200-mesh nylon sieve to prepare a lymphocyte suspension. Next, the suspension was placed into a 15 mL centrifuge tube followed by slow addition of 1 mL of cold RPMI 1640 medium. After centrifugation at 2000 × g for 10 min, the white intermediate layer was collected, and cells were incubated in 5 mL of red blood cell lysis buffer (0.15 mM Tris-NH_4Cl solution) for 2 min, then washed twice with cold PBS. Finally, cells were resuspended in RPMI-1640 media and quantified to 2×10^6 cells/mL. To analyse T lymphocyte differentiation, cells were cultured at 5×10^6 cells/well in 6-well plates stimulated with inactivated bacterial antigens for 24 h, followed by treatment with 2 mM monensin (Sigma-Aldrich, St. Louis, MO, USA) to block intracellular protein transport. Cells were labelled with PerCP-Cy5.5 anti-mouse CD3e antibody, FITC anti-mouse CD4 antibody, and PE anti-mouse CD8a antibody (eBioscience, San Diego, CA, USA) for 45 min and then incubated with APC anti-mouse IFN-γ antibody and PE-Cy7 anti-mouse IL-4 antibody in the presence of Intracellular Fixation and

Permeabilization Buffer (eBioscience, San Diego, CA, USA), as previously described (Qian et al., 2017a, 2017b).

2.8. Protection efficacy against virulent strains challenge in mice

Four weeks after boost, mice were challenged with 5×10^5 CFU of virulent *B. melitensis* 16 M (diluted in 200 μL of PBS) by intraperitoneal injection (Clausse et al., 2013; Qian et al., 2017a). Two weeks later, challenged mice were euthanized to weigh the spleens and calculate the number of *Brucella*. Briefly, spleens were homogenized and serially diluted with PBS, seeded on TBS agar plates, and cultured at 37 °C for four days to count the number of *B. melitensis* 16 M using the mean log CFU ± S.D. per group. Log10 units of protection were obtained by subtracting the mean log CFU/spleen of the immunized group from the mean log CFU/spleen of the PBS group, as previously described (Qian et al., 2017a; Wang et al., 2015).

2.9. Statistics and data analysis

Experimental data between the two groups were analysed by one-way analysis of variance (ANOVA) in SPSS (Version 13.0), and graphs were produced using GraphPad Prism (Version 6) software. In all analyses, a *P*-value of less than 0.05 was considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), while *P*-values greater than 0.05 indicated no significant difference (n.s). All results represent at least four replicates of the experiment. Error bars indicate standard deviation (SD).

3. Results

3.1. Generation of GPI-BCSP31 protein and preparation of VLPs

The *Brucella* BCSP31 sequence enclosed by *Sal* I and *Kpn* I was cloned into rBV, as shown in Fig. 1a. GPI-BCSP31 protein was then expressed through Sf9 infection and further purified using Ni-NTA agarose. The protein was identified with SDS-PAGE and western blotting with an expected molecular mass of approximately 44 kilodaltons (kDa) (Fig. 1b and c). Control VLPs and cVLPs-GPI-BCSP31 were successfully produced using an insect baculovirus expression system. The presence of NDV M protein (40 kDa) in control VLPs and cVLPs-GPI-BCSP31 were proven by western blotting with anti-NDV M polyclonal antibodies (Fig. 1f). In contrast, the existence of BCSP31 was only detected in cVLPs-GPI-BCSP31 and not in control VLPs (Fig. 1g). In terms of direct observation, IEM examination revealed that GPI modification did not alter the morphology of VLPs. Both control VLPs and cVLPs-GPI-BCSP31 exhibited an irregular round shape with surface peplomers at a diameter of approximately 100–200 nm (Fig. 1d and e). The presence of 10 nm gold label on the surface of VLPs also confirmed integration of BCSP31 protein into VLPs (Fig. 1e and Supplemental Fig. 1). The production rate of cVLPs-GPI-BCSP31 generated from the current protocol was 18.9 mg/L ~ 23.5 mg/L, and the purity was 85 ± 7% as calculated

Table 1
Protein concentrations and ratios in VLPs[a].

Protein	1		2		3		Average value ± SD
	μg/mL	Ratio	μg/mL	Ratio	μg/mL	Ratio	
NDV M[b]	227.5	0.65	310.5	0.69	294.0	0.70	0.68 ± 0.03
BCSP31[b]	42.0	0.12	94.5	0.21	75.6	0.18	0.17 ± 0.05
Total protein[c]	350.0	1.00	450.0	1.00	420.0	1.00	1.00
Purity[d]	77%		90%		88%		85 ± 7%

[a] Determined by grey scale of coomassie blue stained polyacrylamide gels.
[b] Determined through BSA concentration-based standard curve.
[c] Total protein concentration was determined by BCA kit.
[d] Calculated through the ratio of BCSP31 and NDV M concentration to total protein concentration.

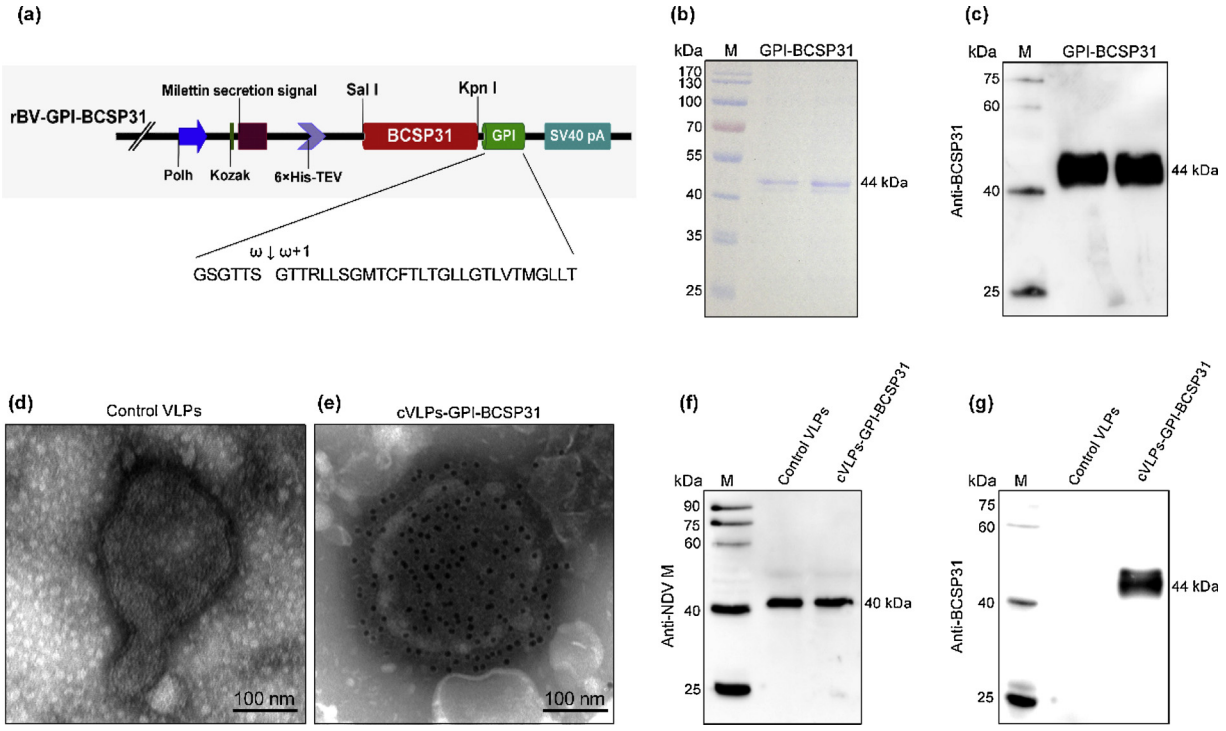

Fig. 1. Production and identification of recombinant protein and VLPs. (a) Construction scheme of a recombinant baculovirus expressing the GPI-BCSP31 protein. (b) SDS-PAGE identification of GPI-BCSP31 expression in a bac-to-bac system with an expected MW of 44 kDa. (c) Western blotting identification of the GPI-BCSP31 protein with anti-BCSP31 polyclonal antibody. (d and e) IEM observation of control NDV VLPs (d) and cVLP-GPI-BCSP31 (e) incubated with anti-BCSP31 primary antibody and gold (10 nm) labelled secondary antibody. (f and g) Western blotting confirmation of cVLPs through detection of NDV M protein (f) and *Brucella* BCSP31 protein (g).

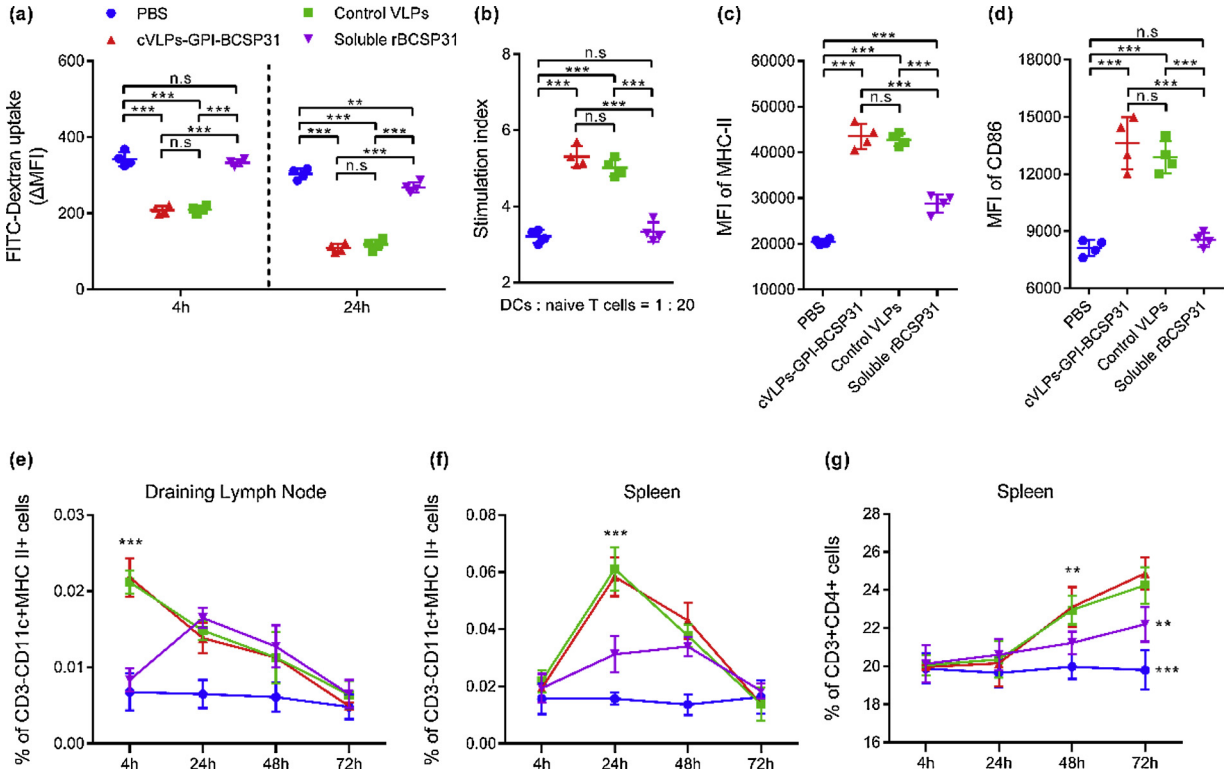

Fig. 2. DC activating ability of VLPs *in vitro* and *in vivo*. (a) Measurement of DC uptake ability alteration after treatment with PBS, control VLPs, cVLPs-GPI-BCSP31, or soluble rBCSP31 for 4 h or 24 h. (b) T cell stimulating ability of differentially (PBS, control VLPs, cVLPs-GPI-BCSP31 or soluble rBCSP31) activated DCs measured by CCK-8 assay. (c and d) DC maturation maker MHC II and CD86 expression levels after stimulation and detection by flow cytometry. (e and f) The presence of DCs in draining lymph nodes (e) and spleens (f) at 4, 24, 48, and 72 h after intravenous administration of PBS, control VLPs, cVLPs-GPI-BCSP31 or soluble rBCSP31 in mice. (g) CD3 + CD4 + cell proliferation in spleen after intravenous administration of PBS, control VLPs, cVLPs-GPI-BCSP31, or soluble rBCSP31 in mice. A *P*-value of less than 0.05 is considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), while a *P*-value greater than 0.05 indicates no significant difference (n.s). All results represent at least four replicates of the experiment. Error bars indicate standard deviation (SD).

through BSA concentration-based standard curve (Table 1 and Supplemental Fig. 2).

3.2. cVLPs-GPI-BCSP31 induced DC activation in vitro and in vivo

To evaluate DC activating conditions after treatment, T cell stimulating ability and maturation surface marker expression were examined *in vitro*, and DC migration and T cell proliferation were assessed *in vivo*. Control VLPs and cVLPs-GPI-BCSP31 treatment led to significantly decreased DC intake ability compared to that of PBS and soluble rBCSP31 groups at 4 h and 24 h (Fig. 2a), suggesting high DC maturation levels. However, soluble rBCSP31 did not influence DC activation at 4 h, while at 24 h, it slightly stimulated DCs (Fig. 2a). In line with this, both control VLPs and cVLPs-GPI-BCSP31 DCs actively stimulated T cell proliferation, indicated by MLR assay (Fig. 2b). These DCs expressed equivalent levels of MHC II and CD86 molecules on the surface, which were significantly higher than PBS and soluble rBCSP31 groups (Fig. 2c and d). Control VLPs and cVLPs-GPI-BCSP31 showed no apparent difference in *in vitro* experiments.

In terms of *in vivo* DC activation, draining lymph nodes (DLNs) and spleens were collected at 4, 24, 48 and 72 h after VLPs or soluble rBCSP31 administration to assess DCs (CD3-CD11c + MHC II + cells) and CD3 + CD4 + T cells. DCs in DLNs of control VLPs and cVLPs-GPI-BCSP31 groups showed a declining trend from 4 h and that in spleens acclimated at 24 h, which then gradually returned to normal levels at 72 h (Fig. 2e and f). Soluble rBCSP31 group DCs in DLNs showed a transient increase at 24 h, and in spleen, they mildly peaked at 48 h (Fig. 2e and f). DCs of the PBS group were consistent from 4 h to 72 h. To confirm the function of migrated DC in spleens, the Th cell population was analysed. CD3 + CD4 + cells of control VLPs and cVLPs-GPI-BCSP31 groups robustly proliferated from 48 h and continued to 72 h (Fig. 2g). Soluble rBCSP31 slightly induced CD3 + CD4 + T cell proliferation, which was significantly weaker than control VLPs and cVLPs-GPI-BCSP31 groups (Fig. 2g).

3.3. cVLPs-GPI-BCSP31 provoked robust humoral cellular immune responses

To confirm its immunogenicity and to determine the proper dose for vaccination, 1 μg, 5 μg, or 10 μg of cVLPs-GPI-BCSP31 was administered to BALB/c mice, and overall serum *Brucella* IgG titre and BCSP31 specific IgG titre were monitored at 2, 6, and 10 weeks. The 5 μg group induced similar levels of total IgG, with the 10 μg group from week 2 to week 10, being significantly higher than the 1 μg group (Fig. 3a). IgG change was not significant at week 2, while at week 6 and 10 it was quite obvious (Fig. 3a). The results of total IgG were similar to that of BCSP31-specific IgG secretion (Fig. 3b).

To compare its efficiency with a commonly used live bacteria formulated commercial vaccine (M5), cVLPS-GPI-BCSP31, PBS, control VLPs and soluble rBCSP31 were separately administered, and IgG levels were consistently assessed for 10 weeks. As control groups, PBS and control VLPs did not stimulate any *Brucella* related antibody changes throughout the experiment, as expected (Fig. 3c and d). Total IgG of all experiment groups (cVLPs-GPI-BCSP31, soluble rBCSP31 and M5) peaked at week six, with the M5 group being highest followed by the cVLPs-GPI-BCSP31 and soluble rBCSP31 groups (Fig. 3c). For BCSP31 specific IgG, the cVLPs-GPI-BCSP31 group was superior to the other groups, with soluble rBCSP31 being the second and M5 the third, suggesting efficacy of cVLPs-GPI-BCSP31 in inducing a BCSP31-specific humoural immune response (Fig. 3d).

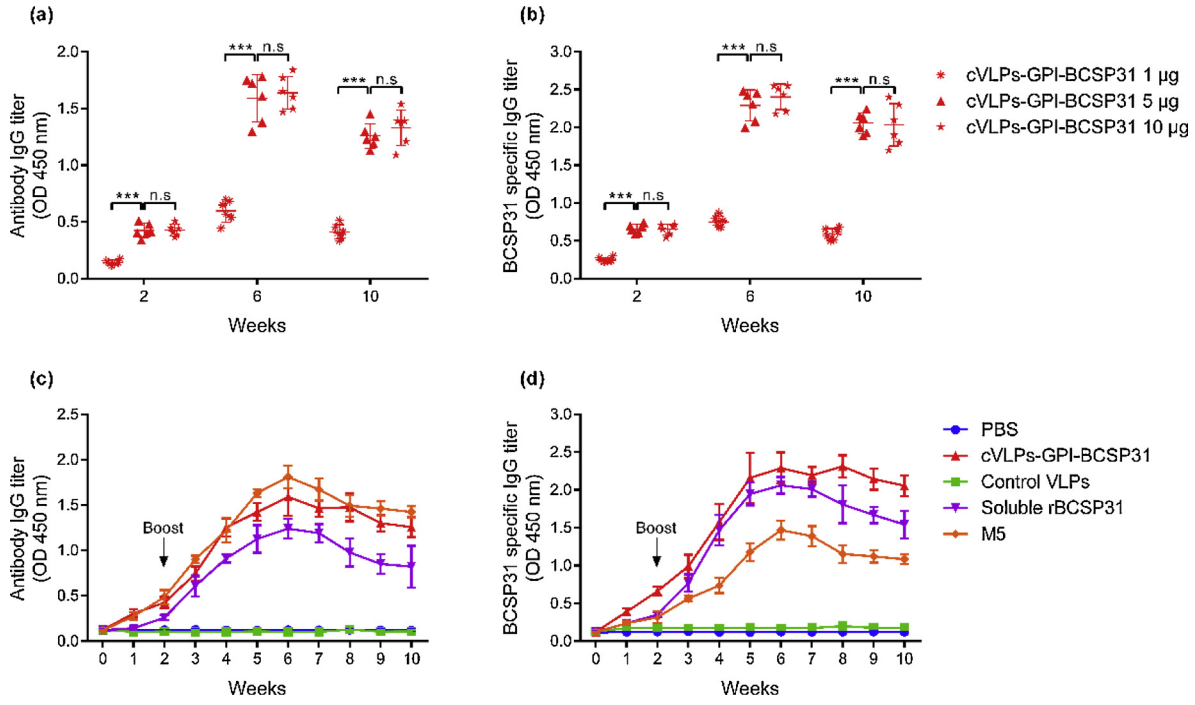

Fig. 3. Antibody response in immunized mice. (a) Serum specific IgG levels of immunized mice with 1 μg, 5 μg, and 10 μg cVLPs-GPI-BCSP31 immunization at 2, 6, and 10 weeks, respectively. (b) BCSP31 protein was coated, and serum from differentially immunized mice at 2, 6, and 10 weeks was analysed for BCSP31 protein-specific IgG antibodies. (c and d) Serum specific IgG level change 10 weeks after immunization with PBS, control VLPs, cVLPs-GPI-BCSP31, soluble rBCSP31, or M5, detected by inactivated *Brucella* (c) or rBCSP31 protein (d) coated ELISA. The Brucella M5 strain group was not subjected to boost. A *P*-value of less than 0.05 is considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), while *P*-values greater than 0.05 indicates no significant difference (n.s). All results represent at least six replicates of the experiment. Error bars indicate standard deviation (SD).

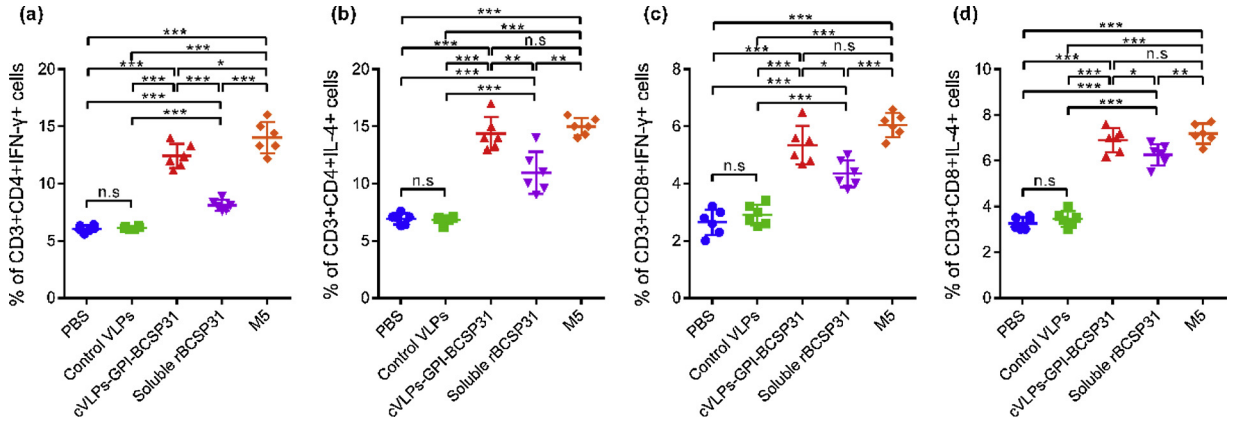

Fig. 4. Cellular immunity in immunized mice. (a and b) Intracellular IFN-γ (a) and IL-4 (b) levels of splenic CD3 + CD4 + cells from immunized mice analysed 6 weeks after primary immunization with PBS, control VLPs, cVLPs-GPI-BCSP31, soluble rBCSP31, or M5 by flow cytometry. (c and d) Intracellular IFN-γ (c) and IL-4 (d) levels of splenic CD3 + CD8 + cells from immunized mice were analysed at week 6. A *P*-value less than 0.05 is considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), while a *P*-value was greater than 0.05 indicates no significant difference (n.s). All results represent at least six replicates of the experiment. Error bars indicate standard deviation (SD).

Cellular immune activity was manifested by differentially proliferating T cell populations, including CD3 + CD4 + IFN-γ + (Th1) cells, CD3 + CD4 + IL-4 + (Th2) cells, CD3 + CD8 + IFN-γ + (Tc1) cells, and CD3 + CD8 + IL-4 + (Tc2) cells. T cell populations of different subtypes displayed a similar pattern among groups. Namely, PBS and control VLPs were significantly lower than the other three experiment groups, while cVLPs-GPI-BCSP31 and M5 groups exhibited high proliferation, significantly better than the soluble rBCSP31 group (Fig. 4a-d). Th2, Tc1, and Tc2 levels were equivalent between the cVLPs-GPI-BCSP31 and M5 groups, while Th1 in M5 was slightly higher than in the cVLPs-GPI-BCSP31 group. Therefore, cVLP-stimulated T cell differentiation was comparable to that of the attenuated live vaccine.

3.4. cVLPs-GPI-BCSP31 conferred sufficient protection against virulent B. melitensis challenge

As cellular and humoural immune response were confirmed, protection efficacy was further tested through bodyweight change (BW), spleen index, and spleen bacteria load (CFU/spleen). After *B. melitensis* 16 M infection, BW of PBS and control VLPs groups dropped for six days and bounced back gradually until day 14 but remained lower than experimental groups to some extent (Fig. 5a). Mice receiving cVLPs-GPI-BCSP31 and M5 continuously grew to the end of the experiment with no detectable behavioural alteration, while the soluble rBCSP31 group lost BW for four days and was gradually restored, while still

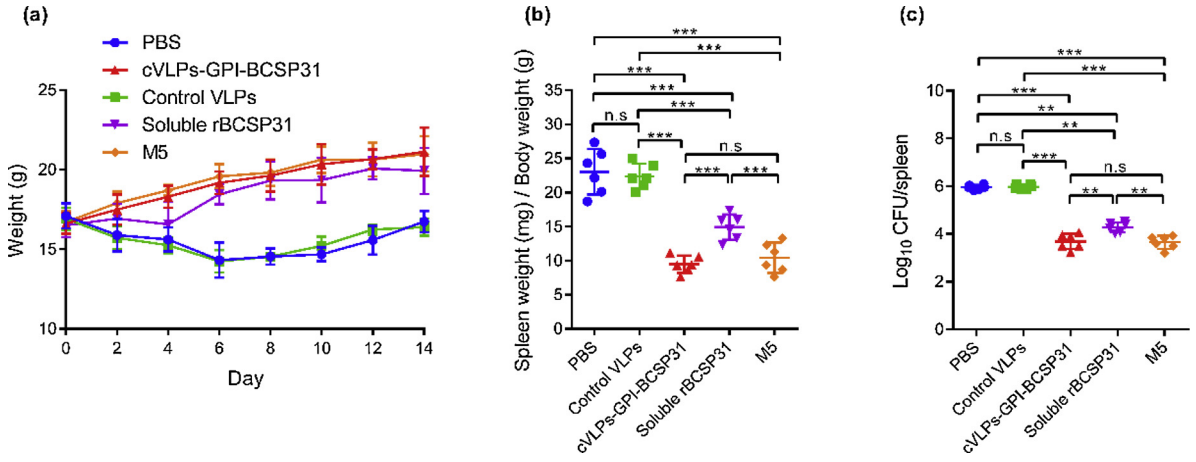

Fig. 5. Protection efficacy against virulent *B. melitensis* strain in immunized mice. (a) Body weight changes of differentially immunized mice within two weeks after virulent *B. melitensis* 16 M challenge. (b) Splenic weight/body weight (spleen index) comparisons of differentially challenged mice were determined. (c) Splenic *B. melitensis* population in differentially immunized mice after challenge were analysed. A *P*-value of less than 0.05 is considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$), while a *P*-value greater than 0.05 indicates no significant difference (n.s). All results represent at least six replicates of the experiment. Error bars indicate standard deviation (SD).

being lower than the cVLPs-GPI-BCSP31 and M5 groups (Fig. 5a).

B. melitensis infection led to severe spleen swelling in PBS and control VLPs groups, which was significantly higher than the other three treatments (Fig. 5b). Among experimental groups, compared with cVLPs-GPI-BCSP31 and M5 groups, soluble rBCSP31 exhibited increased spleen weight to only a limited extent (Fig. 5b). As to spleen colony counts, cVLPs-GPI-BCSP31 and M5 treated mice exhibited the lowest *Brucella* replication, while that of the soluble rBCSP31 group was ten-fold greater. Comparable to M5 (2.31), cVLPs-GPI-BCSP31 (2.27) provided high protection against *B. melitensis* and exhibited equivalent spleen index and protection units (Fig. 5c and Table 2). In general, cVLPs-GPI-BCSP31 performs better than soluble rBCSP31, showing a similar protection ability to M5.

4. Discussion

NDV VLPs are attractive vaccine candidates and foreign protein platforms due to their well-preserved antigen structure and high assembly efficacy (Mcginnes et al., 2011; Pantua et al., 2006). They have been utilized for the delivery of Nipah virus G protein, RSV G, and F protein through the replacement of correspondent compartments of NDV F and HN protein (Mcginnes et al., 2011; Murawski et al., 2010). *Brucella* antigen BCSP31 is not suitable to fuse with the transmembrane domain of NDV HN or F protein, thus it cannot be inserted in VLPs through the aforementioned strategy. Therefore, we exploited a GPI mediated method to attach BCSP31 onto NDV VLP. GPI-anchored proteins must have N-terminal and C-terminal signal peptides to facilitate the transfection process (Chen et al., 2001). Hence, we added the mimetin signal peptide to the fusion sequence as an important component to enhance the protein modification and successfully generated a chimeric BCSP31 decorated VLP. The cVLPs-GPI-BCSP31 was observed to have a typical NDV particle appearance, showing that GPI modification did not influence NDV VLPs structure. Of note, authentic NDV particles revealed pleomorphic structures that were roughly spherical with diameters of approximately 100–500 nm (Yusoff and Wen, 2001); the corresponding NDV VLPs are of various shapes and diameters. Therefore, it exhibited different shapes between Fig. 1d and e. However, both pure VLPs and BCSP31 decorated VLPs showed an M protein constructed envelope and host-derived membrane with no genome content. The results of western blotting against NDV M protein and BCSP31 proved their co-existence in the cVLPs-GPI-BCSP31, while further IEM observation directly confirmed the abundant load of BCSP31 protein on the surface of NDV VLPs. Although cVLPs-GPI-BCSP31 displayed similar **gold** particle densities, it is hard to evaluated BCSP31 insertion level by counting the number of **gold** particles as the result of uneven sizes of VLPs. Therefore, individual protein concentrations in cVLPs were quantified using SDS-PAGE (Table 1 and Supplemental Fig. 2).

Table 2
Protection against *B. melitensis* 16 M in BALB/c mice.

Group	Log_{10} *B. melitensis* at spleen	Log units protection
PBS	5.96 ± 0.09	–
Control VLPs	5.97 ± 0.11	−0.01
cVLPs-GPI-BCSP31	3.69 ± 0.32	2.27[a,b]
Soluble rBCSP31	4.28 ± 0.20	1.68[a]
M5	3.65 ± 0.27	2.31[a,b]

[a] Significantly different compared to PBS controls group ($p < 0.001$).
[b] Significantly different compared to soluble rBCSP31 group ($p < 0.01$).

The insect cell is an effective system for VLP production. In our study, the exogenous gene was codon-optimized to adapt to the insect cell system, and recombinant baculoviruses were screened through plaque formation assay to obtain high production strains (8.8×10^9 IFU/mL for rBV-M, 6.4×10^9 IFU/mL for rBV-GPI-BCSP31). Production conditions were optimized in terms of infection ratio and period in a suspension Sf9-based system. The combination of these improvements contributes to a relatively high production rate and further modification of purification process, allowing the production rate to reach 18.9 mg/L ~ 23.5 mg/L in a 1 L-culture -medium system, comparable to other VLPs (Arevalo et al., 2016). Meanwhile, the purity was 85 ± 7%. These results are promising for future large-scale production.

DCs are crucial during infection, as they are the only APCs that activate primary T cells due to their unique presentation capability (Lee and Iwasaki, 2007). The RSV protein decorating NDV VLPs displays terrific immunogenicity and protection ability through modulation of specific subsets of DCs and effector T cells, which is pivotal for efficient cellular immunity and clearance of intracellular pathogens, such as virus (Kim et al., 2015). In line with this, our previous work also indicated that NDV VLPs can stimulate DC maturation, promoting their migration and ultimate dictating T cell differentiation, both *in vivo* and *in vitro* (Qian et al., 2017b). Active interaction with DCs make NDV VLPs great vehicles for small or soluble antigens by assisting their presentation to APCs. Consistent with early reports, cVLP-GPI-BCSP31 also effectively induce DC activation, as shown by the proliferation of T cells and upregulation of MHC II and CD86 molecules *in vitro*. *In vivo*

migration of DCs from DLNs to the spleen via afferent lymphatics and high endothelial venules was also detected in the cVLPs-GPI-BCSP31 group in this study. As an appropriate T cell differentiation pattern is pivotal for the prevention of intracellular bacteria, splenic T cell subtypes were analysed. It was found that T cell proliferation levels of the cVLPs-GPI-BCSP31 group were significantly higher than those in the soluble rBCSP31 group. This is not ideal for stimulating an IFN-γ and IL-12 mediated T helper type 1 (Th1) response, which is required for the clearance of intracellular pathogens (Oliveira et al., 2011).

For immunization efficacy, our results demonstrated that mice immunized with cVLPs-GPI-BCSP31 exhibited enhanced *Brucella* and BCSP31 specific serum IgG levels compared to soluble rBCSP31-immunized mice. The peak *Brucella* specific IgG levels of the cVLPs-GPI-BCSP31 group were lower than in the M5 group, but the peak period was sustained for far longer. Additionally, the T cell differentiation state of cVLPs-GPI-BCSP31 was comparable to that of attenuated live vaccine (M5). As the T cell-mediated cellular immune response contributes largely to the clearance of intracellular pathogen, the similarities of T cell proliferation between the cVLPs-GPI-BCSP31 and M5 groups may explain the satisfying protection ability against *B. melitensis* invasion. The cVLPs-GPI-BCSP31 group not only exhibited equivalent BW change to the M5 group but also showed decreased spleen swelling and splenic bacterial replication compared to the soluble rBCSP31 group. Another point may also be worth explaining: during the production of NDV VLPs, Sf9 cell or baculovirus component was hard to eliminate. However, our previous study found it did not interfere with the antigen-specific immune response or the DC activation process (Qian et al., 2017b), thus it was not taken into consideration in this study.

5. Conclusions

In summary, we have demonstrated that GPI-BCSP31 proteins successfully anchor onto NDV VLPs and do not alter the morphology of VLPs. The GPI mediated BCSP31 modification also did not interfere with its DC activating ability, indicated by equivalent antigen uptake, antigen-presenting ability, and surface marker expression compared to control VLPs. Furthermore, cVLPs-GPI-BCSP31 elicited strong and effective specific humoural and cellular immune responses, providing sufficient protection against *B. melitensis* challenge, comparable to that of live vaccine M5. Therefore, GPI-BCSP31 anchored NDV VLPs is a safe and effective vaccine candidate against virulent *Brucella*, and the GPI mediated decoration method further extends the application of NDV VLPs based vaccine platform.

Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

X.X, J.L, J.Q and Z.D conceived and designed the experiment. X.X, J.L, Z.B and J.Q performed the experiments. J.D, Y.Y, R.Y and J.Q analysed the data. Z.B, Y.Y, R.Y, X.L, X.W, J.Q and Z.D contributed reagents/materials/analysis tools. X.X, J.L, and J.Q wrote the paper. X.L, X.W, J.Q and Z.D requested financial support. All authors read and approved the manuscript.

Acknowledgements

This work was supported by grants from the National Key Research and Development Program of China (Grant Nos. 2018YFD0500100, 2018YFD0500900, 2016YFD0500900), Jiangsu Provincial Natural Science Foundation of China (Grant Nos. BK20180299), the National Natural Science Foundation of China (Grant Nos. 31772735, 31472195), and Key Technology Research and Development Project of Jilin Province (Grant Nos. 20180201021NY).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.007.

References

Archambaud, C., Salcedo, S.P., Lelouard, H., Devilard, E., De, B.B., Van, R.N., Gorvel, J.P., Malissen, B., 2010. Contrasting roles of macrophages and dendritic cells in controlling initial pulmonary Brucella infection. Eur. J. Immunol. 40, 3458–3471.
Arevalo, M.T., Wong, T.M., Ross, T.M., 2016. Expression and purification of virus-like particles for vaccination. J. Vis. Exp. Jove 54041.
Avila-Calderón, E.D., Lopez-Merino, A., Sriranganathan, N., Boyle, S.M., Contreras-Rodríguez, A., 2013. A history of the development of Brucella vaccines. Biomed. Res. Int. 2013, 743509.
Blasco, J.M., Marín, C., Bagüés, M.P.J.D., Barberán, M., 1993. Efficacy of Brucella suis strain 2 vaccine against Brucella ovis in rams. Vaccine 11, 1291–1294.
Bricker, B.J., Tabatabai, L.B., Deyoe, B.L., Mayfield, J.E., 1988. Conservation of antigenicity in a 31-kDa Brucella protein. Vet. Microbiol. 18, 313–325.
Chen, R., Knez, J.J., Merrick, W.C., Medof, M.E., 2001. Comparative efficiencies of C-terminal signals of native glycophosphatidylinositol (GPI)-anchored proproteins in conferring GPI-anchoring. J. Cell Biochem. 84, 68–83.
Clausse, M., Díaz, A.G., Ghersi, G., Zylberman, V., Cassataro, J., Giambartolomei, G.H., Goldbaum, F.A., Estein, S.M., 2013. The vaccine candidate BLSOmp31 protects mice against Brucella canis infection. Vaccine 31, 6129–6135.
Crawford, R.P., Adams, L.G., Ficht, T.A., Templeton, J.W., Williams, J.D., 1991. Effect of stage of gestation on efficacy of Brucella abortus strain-19 vaccination in cattle. Am. J. Vet. Res. 52, 1848–1851.
Hanson, G.W., 2006. Brucellosis in humans and animals. Ceylon Med. J. 52, 66.
Kim, K.H., Lee, Y.T., Hwang, H.S., Kwon, Y.M., Kim, M.C., Ko, E.J., Lee, J.S., Lee, Y., Kang, S.M., 2015. Virus-like particle vaccine containing the F protein of respiratory syncytial virus confers protection without pulmonary disease by modulating specific subsets of dendritic cells and effector T cells. J. Virol. 89, 11692–11703.
Kim, W.K., Moon, J.Y., Kim, S., Hur, J., 2016. Comparison between immunization routes of live attenuated salmonella typhimurium strains expressing BCSP31, Omp3b, and SOD of Brucella abortus in murine model. Front. Microbiol. 7, 550.
Lee, H.K., Iwasaki, A., 2007. Innate control of adaptive immunity: dendritic cells and beyond. Semin. Immunol. 19, 48–55.
Li, J.Y., Yuan, L., Gao, X.X., Xiang, G., Hong, C., 2014. TLR2 and TLR4 signaling pathways are required for recombinant Brucella abortus BCSP31-induced cytokine production, functional upregulation of mouse macrophages, and the Th1 immune response in vivo and in vitro. Cell Mol. Immun. 11, 477–494.
Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., Bachmann, M.F., 2010. Nanoparticles target distinct dendritic cell populations according to their size. Eur. J. Immunol. 38, 1404–1413.
Mcginnes, L.W., Pantua, H., Laliberte, J.P., Gravel, K.A., Jain, S., Morrison, T.G., 2010. Assembly and biological and immunological properties of Newcastle disease virus-like particles. J. Virol. 84, 4513–4523.
Mcginnes, L.W., Gravel, K.A., Finberg, R.W., Kurtjones, E.A., Massare, M.J., Smith, G., Schmidt, M.R., Morrison, T.G., 2011. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. J. Virol. 85, 366–377.
Mp, J.D.B., Iturralde, M., Arias, M.A., Pardo, J., Cloeckaert, A., Zygmunt, M.S., 2014. The new strains Brucella inopinata BO1 and Brucella species 83-210 behave biologically like classic infectious Brucella species and cause death in murine models of infection. J. Infect. Dis. 210, 467–472.
Murawski, M.R., Mcginnes, L.W., Finberg, R.W., Kurtjones, E.A., Massare, M.J., Smith, G., Heaton, P.M., Fraire, A.E., Morrison, T.G., 2010. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with No evidence of immunopathology. J. Virol. 84, 1110–1123.
Oliveira, S.C., Giambartolomei, G.H., Cassataro, J., 2011. Confronting the barriers to develop novel vaccines against brucellosis. Exp. Rev. Vaccines 10, 1291–1305.
Olsen, S.C., Johnson, C.S., 2012. Efficacy of dart or booster vaccination with strain RB51 in protecting bison against experimental Brucella abortus challenge. Clin. Vaccine Immunol. Cvi 19, 886–890.
Pantua, H.D., Mcginnes, L.W., Peeples, M.E., Morrison, T.G., 2006. Requirements for the assembly and release of Newcastle disease virus-like particles. J. Virol. 80, 11062–11073.
Patel, J.M., Kim, M.C., Vartabedian, V.F., Lee, Y.N., He, S., Song, J.M., Choi, H.J., Yamanaka, S., Amaram, N., Lukacher, A., 2015. Protein transfer-mediated surface engineering to adjuvantate virus-like nanoparticles for enhanced anti-viral immune responses. Nanomed. Nanotechnol. Biol. Med. 11, 1097–1107.
Qian, J., Bu, Z., Lang, X., Yan, G., Yang, Y., Wang, X., 2017a. A safe and molecular-tagged brucella canis ghosts confers protection against virulent challenge in mice. Ve. Microbiol. 204, 121–128.
Qian, J., Xu, X., Ding, J., Yin, R., Sun, Y., Cong, X., Wang, J., Chan, D., Yu, S., Liu, X., 2017b. Newcastle disease virus-like particles induce DC maturation through TLR4/NF-κB pathway and facilitate DC migration by CCR7-CCL19/CCL21 axis. Vet. Microbiol. 203, 158–166.
Roldão, A., Mellado, M.C., Castilho, L.R., Carrondo, M.J., Alves, P.M., 2010. Virus-like particles in vaccine development. Exp. Rev. Vaccines 9, 1149–1176.

Schroeder, R., London, E., Brown, D., 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc. Nat. Acad. Sci. U. S. A. 91, 12130–12134.
Schurig, G.G., Sriranganathan, N., Corbel, M.J., 2002. Brucellosis vaccines: past, present and future. Vet. Microbiol. 90, 479–496.
Seleem, M.N., Boyle, S.M., Sriranganathan, N., Pfeffer, M., Truyen, U., Gaastra, W., 2010. Brucellosis: a re-emerging zoonosis. Vet. Microbiol. 140, 392–398.
Verger, J.M., Grayon, M., Zundel, E., Lechopier, P., Olivier-Bernardin, V., 1995. Comparison of the efficacy of Brucella suis strain 2 and Brucella melitensis Rev. 1 live vaccines against a Brucella melitensis experimental infection in pregnant ewes. Vaccine 13, 191–196.
Wang, X., Chang, A., Yang, M., Li, X., Ke, Y., Lei, S., Xu, X., Yu, J., Ren, H., Du, X., 2015. Immunization with individual proteins of the Lrp/AsnC family induces protection against Brucella melitensis 16M challenges in mice. Front. Microbiol. 6, 1193.
Wattam, A.R., Williams, K.P., Snyder, E.E., Shukla Jr, A.N., Dickerman, A.W., Crasta, O.R., Kenyon, R., Lu, J., Shallom, J.M., 2009. Analysis of ten Brucella genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle. J. Bacteriol. 191, 3569–3579.
Yu, D.H., Li, M., Hu, X.D., Cai, H., 2007. A combined DNA vaccine enhances protective immunity against Mycobacterium tuberculosis and Brucella abortus in the presence of an IL-12 expression vector. Vaccine 25, 6744–6754.
Yusoff, K., Wen, S.T., 2001. Newcastle disease virus: Macromolecules and opportunities. Avian Pathol. 30, 439–455.