Virus-like particles of recombinant PCV2b carrying FMDV-VP1 epitopes induce both anti-PCV and anti-FMDV antibody responses

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Abstract

Mixed infection of porcine circovirus type 2 (PCV2) and foot-and-mouth disease virus (FMDV) is devastating to swine populations. To develop an effective vaccine that can protect the pigs from the infection of PCV2 and FMDV, we used the neutralizing B cell epitope region (aa 135–160) of FMDV to replace the regions aa 123–151 and aa 169–194 of the PCV2b Cap protein to generate a recombinant protein designated as Capfb. The Capfb protein was expressed in *Escherichia coli* system and the purified Capfb protein assembled into virus-like particles (VLPs) through dialysis. The ability of the Capfb protein to induce effective immune response against FMDV and PCV2b was tested in mice and guinea pigs. The results showed that the Capfb-VLPs could elicit anti-PCV2b and anti-FMDV antibody response in mice and guinea pigs without inducing antibodies against decoy epitope. Moreover, the Capfb-VLPs could enhance the percentage and activation of B cells in lymph nodes when the mice were stimulated with inactivated FMDV or PCV2b. These data suggested that the Capfb-VLPs could be an efficacious candidate antigen for developing a novel PCV2b-FMDV bivalent vaccine.

Keywords Porcine circovirus type2 · Foot-and-mouth disease virus · Vaccine · Virus-like particle · Antibody response

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Introduction

Porcine circovirus type 2 (PCV2), a member of the *Circovirus* genus of the *Circoviridae*, is a small non-enveloped virus containing a circular single-stranded DNA genome with 1.76-kb length (Huang et al. 2011), which mainly encodes replicase protein and capsid (Cap) protein. The Cap protein encoded by open reading frame 2 (ORF2) can independently assemble into virus-like particles (VLPs) and is major immunogen for vaccine development (Mankertz et al. 2004; Nawagitgul et al. 2000). PCV2 is considered to be one of the most important pathogens essentially in all major swine-producing countries (Gillespie et al. 2009), since it could cause PCV-associated disease (PCVAD) which includes wasting, increased mortality, respiratory signs, enteritis, reproductive failure, and porcine dermatitis and nephropathy syndrome (PDNS) in pigs (Opriessnig et al. 2007). Moreover, immunosuppression triggered by PCV2 could make infected animals susceptible to other pathogens (Allan et al. 2004) usually resulting in mixed infections. However, the commercial PCV2

vaccines could only prevent the pigs from PCV2 (Beach and Meng 2012) instead of the mixed infection.

Foot-and-mouth disease virus (FMDV) is one of the most devastating viruses to swine populations when the pigs are co-infected with PCV2 (Allan et al. 2004). It is highly infectious and could cause foot-and-mouth disease (FMD) in over 70 cloven-hoofed species such as cattle, sheep, and swine (Grubman and Baxt 2004; Stenfeldt et al. 2014). Infected animals exhibit a high fever, followed by blister-like sores in the mouth and on the feet resulting in lameness and increased death rate (Alexandersen et al. 2003). FMDV is a single-stranded, positive-sense RNA virus belonging to Aphthovirus genus and contains a capsid composed of four structural proteins identified as VP1, VP2, VP3, and VP4 of FMDV (Doel and Collen 1982). Among these proteins, VP1 can induce neutralizing antibodies in hosts through the immunogenic site located in the G-H loop (Saiz et al. 2002). The G-H loop is a protruding element on the surface of the FMDV capsid spanning about 20 residues around positions 140–160 (Kupper et al. 1981; Pfaff et al. 1982) and contains a highly conserve Arg-Gly-Asp (RGD) motif which interacts with cell surface integrin receptors and contributes to the induction of neutralizing antibodies (Jackson et al. 2002; Verdaguer et al. 1996). A synthetic peptide comprising amino acid (aa) 140–160 of the type O FMDV-VP1 protein could induce the production of neutralizing antibodies in guinea pigs (Bittle et al. 1982). Another recombinant vaccine involving aa 141–160 of the type O FMDV-VP1 protein and the swine immunoglobulin G heavy-chain constant region against type O FMDV could also induce high level of anti-FMDV-specific antibodies in swine and provide protection (Shao et al. 2011). Thus the G-H loop is an important immunogenic region for developing novel vaccines against FMDV.

The subunit vaccine based on VLPs is not only effective to stimulate immune response but also non-infectious and can be easily produced on a large scale (Crisci et al. 2012; Zeltins 2013). Therefore, many types of VLPs have been tried as carriers for foreign epitopes to induce antibody responses against target antigens. VLPs formed by VP2 of porcine parvovirus (PPV) carrying CD8⁺ T cell epitope of the lymphocytic choriomeningitis virus (LCMV) in the N-terminus can induce strong responses of cytotoxic T lymphocyte (CTL) which could kill virally infected cells, and confer total protection against LCMV challenge in mice (Andersen et al. 2006) (Sedlik et al. 1997). VLPs formed by PCV2 have also been successfully used as foreign epitope carriers (Zhang et al. 2014). Chimeric VLPs of PCV2 generated by replacing the nuclear localization signal (NLS) of PCV2 Cap protein with classical swine fever virus (CSFV) T cell epitope and B cell epitope could form VLPs and induce efficient humoral and cellular immunity against PCV2 and CSFV in mice (Zhang et al. 2014). However, the decoy epitope (aa 169–180) recently identified in the Cap of PCV2 has been demonstrated to divert

the antibody response away from protective epitopes (Trible et al. 2012). The decoy epitope forms an external loop structure protruding from the surface in the Cap monomer, while it is buried near the interface of the 3-fold axis in the icosahedral PCV2 particles (Trible et al. 2012). Therefore, the antibodies induced by this epitope display low neutralizing activity and could not confer protection. Up to now, four major genotypes of PCV2 have been identified: PCV2a, PCV2b, PCV2c, and PCV2d, and among them, PCV2b is the most widespread genotype in recent years (Franzo et al. 2016).

Thus, to develop an effective vaccine that can protect the pigs from the mixed infection of PCV2 and FMDV, the neutralizing B cell epitope region (aa 135–160) containing G-H loop of type O FMDV-VP1 protein was used to replace the region (aa 169–194) involving the decoy epitope (aa 169–180) and a protruding region (aa 123–151) of the PCV2b Cap protein to generate a recombinant protein designated as Capfb. The Capfb protein expressed in the *Escherichia coli* system was assembled into VLPs in vitro and its efficacy was subsequently evaluated in mice and guinea pigs.

Materials and methods

Protein structure prediction

The secondary and 3D structures of the recombinant protein were predicted by DNASTA Protean software and the Swiss Model website (<http://swissmodel.expasy.org/>), respectively. The PyMOL software was used to visualize 3D structures.

Construction of recombinant expression plasmid

The recombinant pET28a plasmid encoding Cap protein of PCV2b with sixhistidine tag (pET28a-CP-His) was constructed in our lab as previously described (Yu et al. 2016). Using the pET28a-CP-His as a template, fragments A, B, and C were amplified by PCR with the primers shown in Table 1 (Fig. S1A). Then the fragments A and B were cloned into pET28a plasmid with the modified multiple cloning sites using *Nco*I and *Eco*RI and *Eco*RI and *Bam*HI, respectively (Fig. S1B). Finally, the resultant plasmid digested with *Bam*HI and *Xho*I was ligated with the fragment C digested with *Bgl*II and *Xho*I sites to obtain the pET28a-Capfb-His plasmid which could encode the Capfb protein (Fig. S1B).

Expression and purification of recombinant protein

The pET28a-Capfb-His were transformed into *E. coli* BL21 (DE3) (Invitrogen) for expressing the Capfb protein under IPTG induction. Then the Capfb protein was purified by nickel affinity chromatography (GE Healthcare, UK). The supernatant from the lysate of the *E. coli* was loaded onto the nickel

Table 1 Primers used in this study

Genes	Names	Primer sequence (5'–3')	Restriction site
Fragment A	1F	CCATGGGTATGACTTATCCACGTCG	<i>NcoI</i>
	1R	GAATTCGACCTTCACCTTGC GGATG	<i>EcoRI</i>
Fragment B	1F	CTCCAAATACGCTGAAGTTCTCTGACCAACGTCCGTGGTGACCTTCAAGTATTGGCTC	
	1R	GGTAAGAAAACGGCTGCGGAATTGGAAGTGGACGAGCAGCCTTCTGAGCCAATACTTGA	
	2F	GAATTCTGGCCATGTTCTCTATTACTCAAGGTGATCGTGGTGTCGGCTCCAAATACGC	<i>EcoRI</i>
	2R	GGATCCTTCAGCGTATTTGACTGGTTTCGGAGTGAAATAACGGGAGTGGTAAGAAAACG	<i>BamHI</i>
Fragment C	1F	AAGTATTGGCTCAGAAGGCTGCTCGTCCACTTCCAGTTGATCACGTCCGACTT	
	1R	CTCGAGCTATTAGTGGTGGT	<i>XhoI</i>
	2F	AGATCTCTGACCAACGTCCGTGGTGACCTTCAAGTATTGGCTCAGAAG	<i>BglII</i>
	2R	CTCGAGCTATTAGTGGTGGT	<i>XhoI</i>

The sequences in bold represent the restriction sites

F forward primer, R reverse primer

affinity column. Unbound proteins were removed by washing the column with washing buffer (20 mM Tris, 500 mM NaCl, 30 mM imidazole, 10 mM β -mercaptoethanol, 5% glycerol, 0.05% Tween 80, pH 8.0) and the bound Capfb protein was eluted in an elution buffer (20 mM Tris, 500 mM NaCl, 500 mM imidazole, 50 mM β -mercaptoethanol, 5% glycerol, 0.05% Tween 80, pH 8.0). The Capfb protein expressed in *E. coli* and purified by nickel affinity chromatography was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot

The *E. coli* lysates were subjected to 12% SDS-PAGE and then transferred onto nitrocellulose membrane (Whatman, Germany) using TE 77 PWR Semi-Dry Transfer Unit (GE Healthcare, UK). Specific antibody raised from ICR mice immunized with inactivated PCV2b or FMDV (1:100 dilution) were used to identify the Capfb protein.

Transmission electron microscopy

The purified Capfb protein was dialyzed against assembly buffer (50 mM Na_2HPO_4 , 50 mM NaH_2PO_4 , 500 mM NaCl, 5% glycerol, 0.03% Tween 80, 1% Triton X-100, pH 6.5) to yield assembled VLPs. The VLPs were observed under transmission electron microscopy (TEM) (FEI, Tecnai Spirit, the Netherlands) at an accelerating voltage of 80 kV and photographed at a magnification of 40,000. The Cap protein of PCV2b expressed and assembled as previously described (Yu et al. 2016) was used as a control.

Vaccine preparation

The Capfb-VLPs or the Cap-VLPs were formulated with ISA 35 adjuvant, an oil-based adjuvant of oil-in-water (O/W)

emulsion (Seppic®, France), in a 3:1 volume ratio (antigen volume:adjuvant volume) for preparing Capfb or Cap vaccine.

Animals and immunizations

Animal experiments were performed according to the protocols approved by the Scientific Investigation Board of Science & Technology of Jilin Province and in accordance with the National Institute of Health Guide for the Care. Specific pathogen-free female ICR mice (20 $\pm$ 1 g) and female guinea pigs (about 300 g) were obtained from the Experimental Animal Center, Medical College of Norman Bethune, Jilin University.

ICR mice, six in each group, were intramuscularly immunized with Capfb or Cap vaccine (10 μ g/mouse) on days 0 and 14, and bled on days 0, 28, and 35 for isolating serum. Guinea pigs, four in each group, were intramuscularly immunized with Capfb or Cap vaccine (20 μ g/guinea pig) on days 0, 14, and 28, and bled on days 0 and 42 for isolating serum.

Enzyme-linked immunosorbent assay

Inactivated FMDV or PCV2b was used to detect the FMDV-specific or PCV2b-specific antibodies in the sera of mice and guinea pigs immunized with Cap vaccine or Capfb vaccine. The D3 protein, a recombinant protein composing of nine copies of the decoy epitope (aa 169–180) of PCV2 Cap protein expressed in *E. coli* system (Yu et al. 2016), was used as coating antigen to detect the PCV2b decoy epitope specific antibodies in the sera of mice immunized with Capfb vaccine or Cap vaccine. The levels of specific antibody were expressed as Optical densities (OD) at 492 nm $\pm$ standard deviation (SD).

Stimulation and flow cytometry assay

ICR mice were intramuscularly immunized with 100 μ l of Capfb vaccine (10 μ g/mouse) or NaCl on days 0 and 14, stimulated with inactivated FMDV or PCV2b (10 μ g/mouse) on day 21, and sacrificed on day 22 for isolating lymph node cells on the same side as the injection site. The lymph node cells were then stained with PE-conjugated anti-CD19 and FITC-conjugated anti-CD69 antibodies for 30 min on ice in dark, washed with PBS, and detected by flow cytometry.

Statistical analysis

The results were analyzed using *t* test by SPSS 19.0 software for Windows. All data were expressed as means $\pm$ standard deviation (SD). The significance level was set at 0.05, and *P* < 0.05 was considered statistically significant.

Results

Design of the PCV2b capsid protein carrying FMDV epitopes

The neutralizing B cell epitope (aa 135–160) containing G-H loop of type O FMDV-VP1 protein was used to replace the region (aa 169–194) involving the decoy epitope and a protruding region (aa 123–151) of the PCV2b Cap protein to generate a recombinant protein designated as Capfb (Fig. 1a).

To test the effects of replacing the regions (aa 123–151 and aa 169–194) of the Cap protein with the B cell epitopes of FMDV-VP1, the secondary structure, hydrophilicity, and antigenic index of Cap and Capfb were detected with DNASTar Protean software (Fig. 1b). The secondary structures of the B cell epitopes of FMDV-VP1 in the Capfb protein (aa 123–148 and aa 166–191) and their corresponding regions in the Cap protein (aa 123–151 and aa 169–194) were similar, that all of them contained several Alpha, Beta, or Turn structures (Fig. 1b). Compared with the corresponding regions in the Cap protein, the B cell epitope of FMDV-VP1 located in positions aa 123–148 of the Capfb protein displayed reduced hydrophilicity but increased antigenic index, while the B cell epitope of FMDV-VP1 located in aa 169–194 showed reduced hydrophilicity but similar antigenic index (Fig. 1b). These results indicated that the B cell epitopes of FMDV-VP1 in the Capfb protein showed good antigenicity and should be able to stimulate the body to produce antibodies against FMDV.

To further confirm whether the B cell epitope of FMDV-VP1 in the Capfb protein could function well, the 3D structures of Capfb, Cap, and FMDV-VP1 were predicted through the Swiss Model website. We found that the 3D structure of the Cap and the Capfb protein were similar and the B cell epitopes of FMDV-VP1 in the Capfb protein regions (Fig.

1e, left panel) could be exposed on the surface as their corresponding regions (aa 123–151 and aa 169–194) did in the Cap protein (Fig. 1c). Moreover, the RGD motif of the B cell epitopes of FMDV-VP1 in the Capfb protein could also form loop structure (Fig. 1e, right panel) as that in the natural FMDV-VP1 did (Fig. 1d). These results demonstrated that the B cell epitopes of FMDV-VP1 in the Capfb protein could function well and would not affect the Capfb to form VLPs.

Expression, identification, and purification of Capfb

The expression of the Capfb protein in *E. coli* lysates were analyzed by SDS-PAGE and Western-blot using the anti-FMDV or anti-PCV2b specific antibodies as primary antibodies. The SDS-PAGE analysis showed that most of the Capfb protein with molecular weights of about 28.1 kDa existed in the supernatant from the lysate of the *E. coli*, while little in the precipitation, indicating the Capfb were soluble proteins (Fig. 2a). The results of Western-blot revealed that the specific 28.1 kDa band corresponding to that of the Capfb could be recognized by both the anti-FMDV and anti-PCV2b specific antibodies (Fig. 2b). Then the Capfb protein was purified by nickel affinity chromatography. The supernatant from the lysate of the *E. coli* was loaded onto the nickel affinity column and multiple elution fractions were obtained. The Capfb protein with high purity approximately 28.1 kDa in the elution fractions was visible on the SDS-PAGE gel (Fig. 2c).

Transmission electron microscopy analysis of Capfb

To examine whether the Capfb protein can be assembled into VLPs, TEM analysis was performed. The results showed that the purified Capfb protein could not spontaneously form VLPs (Fig. 3a), while after being dialyzed against assembly buffer (50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 500 mM NaCl, 5% glycerol, 0.03% Tween 80, 1% Triton X-100, pH 6.5) in vitro, the Capfb protein appeared to have assembled into particles (Fig. 3b). The assembled Cap protein was also observed under TEM as a control. The Cap protein could form homogenous and tight VLPs (Fig. 3c). In comparison, the Capfb-VLPs were looser than the Cap VLPs. The diameters of VLPs formed by both the Cap and the Capfb protein were 17–20 nm (Fig. 3b, c).

Capfb-induced FMDV-specific and PCV2b-specific antibody responses in mice and guinea pigs

To test whether the Capfb-VLPs could efficiently induce both anti-FMDV and anti-PCV2b antibodies, mice were immunized with Capfb vaccine or Cap vaccine as control, on days 0 and 14 (Fig. 4a). The levels of FMDV and PCV2b specific antibodies in the sera collected on days 0, 28, and 35 were detected by enzyme-linked immunosorbent assay (ELISA)

Fig. 1 Design and structure prediction of Capfb. **a** Schematic diagram of Cap and Capfb. **b** Secondary structure prediction of Cap and Capfb. 3D structure prediction of **c** the Cap protein of PCV2b, **d** the VP1 protein of FMDV, and **e** the Capfb protein. Green, a protruding region (aa 123–151) of the PCV2b Cap protein. Pink, the PCV2b decoy epitope containing region (aa 169–194). Blue, the neutralizing B cell epitope (aa 135–160) of FMDV-VP1. Red, the RGD motif

(Fig. 4a). The Capfb-VLPs could elicit vigorous antibody response against inactivated FMDV and PCV2b on days 28 and 35 (Fig. 4b), while the Cap-VLPs could only induce anti-PCV2b specific IgG since it involved no B cell epitope of FMDV-VP1 (Fig. 4c). Noticeably, the mice inoculated with the Capfb-VLPs produced significantly higher level of anti-

PCV2b specific IgG on day 35 than the mice immunized with the Cap-VLPs ($P = 0.0022$, Fig. 4c). The results demonstrated that the Capfb-VLPs could induce the antibody response against inactivated FMDV and PCV2b. Moreover, the Capfb protein could stimulate the mice to produce higher level of PCV2b-specific antibodies than the Cap-VLPs.

Fig. 2 Expression, identification, and purification of Capfb. **a** SDS-PAGE analysis of Capfb expressed in *E. coli*. Control, lysate of *E. coli* without IPTG induction; total, whole cellular proteins in the lysate of *E. coli*; Sup., the supernatant of the lysate; pellet, the precipitation of the lysate. **b** Western-blot analysis of Capfb expressed in *E. coli*. Primary antibody is the specific antibody raised from the mice immunized with inactivated PCV2b or FMDV and secondary antibody

is the goat anti-mouse IgG/HRP. **c** SDS-PAGE analysis of purified Capfb. Loading represents the lysate of the *E. coli* loaded onto the nickel affinity column. Lanes 1–5 showed the Capfb in the elution fractions 1, 2, 3, 4, and 5 collected from the nickel affinity chromatography, respectively. M represents the molecular weight marker. The arrow denotes the Capfb with the calculated molecular weight of 28.1 kDa

To further observe whether the Capfb protein could induce specific antibody response against FMDV and PCV2b in different species of animals, guinea pigs were vaccinated with Capfb vaccine on days 0, 14, and 28 and were bled on days 0 and 42 for detecting anti-FMDV IgG and anti-PCV2b IgG levels by ELISA (Fig. 4d). The results showed that the anti-FMDV IgG ($P = 0.0305$, Fig. 4e) or anti-PCV2b IgG ($P = 0.0022$, Fig. 4f) could be induced by the Capfb vaccine on days 42, respectively. The results demonstrated that the Capfb-VLPs had the potential to be widely used for vaccination among different species of animals.

Decoy epitope of PCV2b-directed antibody responses in mice

D3 protein containing nine copies of the decoy epitope of PCV2b was used as a coating antigen to detect the anti-decoy epitope antibodies in the sera collected on day 28 of the mice immunized with the Capfb or Cap vaccine. As expected, anti-decoy epitope antibodies were not detected in the sera of mice immunized with the Capfb-VLPs (Fig. 5). Compared with the Capfb-VLPs, Cap-VLPs induced significantly higher level of antibodies against the decoy epitope

Fig. 3 Analysis of Capfb structure by transmission electron microscopy. Electron micrographs of **a** unassembled Capfb protein, **b** assembled Capfb VLPs, and **c** assembled Cap-VLPs at a magnification of 40,000. Bars represent 50 nm

Fig. 4 Capfb-induced antibody response in mice and guinea pigs. Immunization procedure of mice (a) and guinea pigs (d). The FMDV-specific IgG was detected in the sera (1:100 dilution) of the mice (b) or guinea pigs (e) immunized with Cap or Capfb vaccine by ELISA using inactivated FMDV as coating antigens. PCV2b-specific IgG were detected in the sera (1:100 dilution) of the mice (c) or guinea pigs (f) immunized with Capfb vaccine by ELISA using inactivated PCV2b as coating antigen. Each point represents the IgG level of one mouse. Bars show mean $\pm$ SD

($P = 0.001$, Fig. 5), indicating the decoy epitope of Cap-VLPs could be exposed during the process of the vaccination.

Percentage and activation of B cells in lymph node cells of mice after stimulation by inactivated FMDV or PCV2b

To explore whether the Capfb protein could induce immune response when mice were stimulated with FMDV or PCV2b, we immunized the mice with Capfb vaccine or NaCl as control, on days 0 and 14, and stimulated the mice with inactivated FMDV or PCV2b on day 21 (Fig. 6a). Twenty-four hours after the stimulation, we sacrificed the mice and detected the percentage and activation of B cells in the lymph

node on the same side of the injection site (Fig. 6a). The results showed that when mice were stimulated with inactivated FMDV, compared with the mice in the control group, significantly higher percentage of CD19⁺ cells ($P = 0.0149$, Fig. 6b) and CD19⁺ CD69⁺ cells ($P = 0.0464$, Fig. 6c) could be detected in the mice immunized with Capfb vaccine. Similarly, there were obviously higher percentage of CD19⁺ cells ($P = 0.0326$, Fig. 6d) and CD19⁺ CD69⁺ cells ($P = 0.0294$, Fig. 6e) in the mice immunized with Capfb vaccine after stimulation by PCV2b. The results suggested that the Capfb-VLPs could enhance the percentage and activation of B cells in the lymph node when mice were stimulated with inactivated FMDV or PCV2b.

Fig. 5 Decoy epitope of PCV2b-directed antibody responses in mice. Mice were immunized with Cap or Capfb vaccine on days 0 and 14 and bled on day 28. D3 protein composing of nine copies of PCV2b decoy epitope was used as a coating antigen to detect the decoy epitope-directed IgG in the sera (1:100 dilution) of the mice by ELISA. Each point represents the IgG level of one mouse. The dotted line represents the IgG level in negative sera. Bars show mean $\pm$ SD

Discussion

To develop an effective vaccine that can protect the pigs from the mixed infection of PCV2 and FMDV, we used the neutralizing B cell epitope region (aa 135–160) to replace two regions (aa 123–151 and 169–194) of the PCV2b Cap protein to generate a recombinant protein designated as Capfb. The Capfb protein could be assembled into VLPs in vitro and induce the antibody response against both PCV2b and FMDV in mice and guinea pigs.

The VLPs generated through genetic engineering have been used as a platform carrying foreign epitopes to induce multiple immune responses (Casal 1999; Crisci et al. 2012). Chimeric PCV2-VLPs were also reported to be used as antigen vehicle carrying CSFV T cell and B cell epitopes (Zhang et al. 2014). The existing VLPs vehicle carrying foreign epitopes were expressed in baculovirus system (Casal 1999; Sedlik et al. 1997; Zhang et al. 2014). In our study, the *E. coli* system was used to express the Capfb, a recombinant PCV2b Cap protein carrying epitopes of FMDV-VP1. After being dialyzed in vitro, the Capfb protein could also be observed to form VLPs under TEMs. However, the VLPs formed by the Capfb seemed to be looser than that formed by the Cap protein indicating that the replacement of the two regions (aa 123–151 and 169–194) of PCV2b Cap protein with the FMDV-VP1 epitope may slightly influence the formation of the VLPs.

Though the VLPs formed by the Capfb protein did not identically mimic the natural structure of the PCV2b particles,

the Capfb-VLPs still elicited vigorous anti-PCV2b antibody response in mice and guinea pigs. Noticeably, the Capfb-VLPs could induce higher level of anti-PCV2b antibodies than the Cap-VLPs. The enhanced production of anti-PCV2b antibodies induced by the Capfb-VLPs could be correlated with the replacement of the region (aa 169–194) containing decoy epitope. Evidences have demonstrated that the decoy epitope displayed an external loop protruding from the outer surface of the PCV2b Cap monomer, while was buried near the interface of the icosahedral PCV2 particles (Trible et al. 2012). Therefore, the decoy epitope could not induce neutralizing antibodies but diverted the humoral response away from a protective epitope (Trible et al. 2012). Incorrect fold or posttranslational modification of the recombinant protein expressed in *E. coli* system (Bucarey et al. 2009), may cause the exposure of the decoy epitope. In this study, the Cap-VLPs rather than the Capfb-VLPs were found to induce the decoy epitope-directed antibodies in the vaccinated mice. Thus, the replacement of the decoy epitope region in the Capfb protein could avoid the exposure of the decoy epitope and maintain the protective antibody response. Consistently, our previous study also showed that the replacement of the decoy epitope (aa 169–180) with a neutralizing epitope (aa 113–131) in the PCV2b Cap protein could increase the production of the anti-PCV2b antibodies and induces improved protective immune responses in pigs challenged with PCV2b (Yu et al. 2016).

Besides vigorous anti-PCV2b antibody response, the Capfb-VLPs could also induce high level of antibodies which could be recognized by inactivated FMDV in both mice and guinea pigs. Two regions (aa 123–151 and 169–194) of the PCV2b Cap protein were chosen to insert the neutralizing B cell epitope region of FMDV-VP1, since the aa 130, 131, and 169–180 were protruding elements on the surface of the PCV2b Cap protein (Saha et al. 2012; Tribble et al. 2012). We tried to insert the FMDV-VP1 epitope into the aa 130–131 and aa 169–180 with or without their flanks and used DNASTA Protean software and the Swiss Model website to predict their secondary and 3D structures. We found that the FMDV-VP1 epitopes inserted into the regions aa 123–151 and aa 169–194 could protrude from the surface of the Capfb protein and mimic the natural structure, especially the RGD motif which interacts with cell surface integrin receptors and contributes to the induction of neutralizing antibodies (Jackson et al. 2002; Verdaguer et al. 1996). Therefore, we used the FMDV-VP1 epitope to replace the regions aa 123–151 and aa 169–194 of the PCV2b Cap protein. Moreover, the mice immunized with Capfb vaccine had significantly higher percentage and activation of B cells in the lymph nodes when stimulated with inactivated

Fig. 6 Percentage and activation of B cells in lymph node cells of mice after stimulation by inactivated FMDV or PCV2b. **a** Schematic diagram of experimental procedure. Percentage of CD19⁺ cells (**b, d**) and CD19⁺ CD69⁺ cells (**c, e**) in the lymph node cells on the same side of the injection site of mice (four per group) after being stimulated with inactivated FMDV or PCV2b were detected by flow cytometry assay. Bar shows mean $\pm$ SD

FMDV or PCV2b, indicating the Capfb protein had the potential to elicit protective immune response when animals were infected with FMDV or PCV2b. Prospectively, we may use live FMDV or PCV2b to challenge the animals immunized with the Capfb vaccine in high-level biosafety containment organisms to test the neutralizing activity of Capfb.

In the previous study, a recombinant virus PCV2 containing the epitope region (aa 141–160) of FMDV-VP1 in its C-terminal was also demonstrated to be able to induce anti-PCV2-Cap protein antibodies and anti-FMDV-VP1 antibodies in mice (Huang et al. 2014). However, the recombinant virus could replicate and cause various pathological changes in mice (Huang et al. 2014); thus, the vaccines developed

based on it may be infectious and have security risks. Compared with the recombinant virus, the Capfb-VLPs is not only able to elicit vigorous antibody response against PCV2b and FMDV, but also non-infectious since it did not contain viral genome.

In summary, our results suggest that the Capfb, a recombinant PCV2b Cap protein carrying FMDV-VP1 epitopes in aa 123–151 and aa 169–194 expressed in *E. coli* system could form VLPs through dialysis and elicit vigorous anti-PCV2b and anti-FMDV antibody response in mice and guinea pigs. Thus, the Capfb-VLPs could be an efficacious candidate antigen for developing novel PCV2b-FMDV bivalent vaccine.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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