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Fusion of pseudorabies virus glycoproteins to IgG Fc enhances protective immunity against pseudorabies virus

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ABSTRACT

Molecular adjuvants are vaccine delivery vehicle to increase specific antigens effectiveness. Herein, we concentrated on IgG Fc, an effective molecular adjuvant, to develop novel pseudorabies virus (PRV) subunit vaccines. Two major protective antigen genes of PRV were constructed and linked into the mouse IgG Fc fragment. The gD, gD-IgG2aFc, gB and gB-IgG2aFc proteins were expressed using a baculovirus system. Mice intranasally immunized with gD-IgG2aFc or gB-IgG2aFc subunit vaccine exhibited significantly higher PRV-specific antibodies, neutralizing antibodies and intracellular cytokines than the mice intranasally immunized with gD or gB subunit vaccine. Moreover, no histopathological lesions were observed in mice immunized with gB-IgG2aFc subunit vaccine via histopathology examination. Further, the gB-IgG2aFc subunit vaccine was efficient for PRV infection compared with live attenuated vaccine. Overall, these results suggest that IgG2a Fc fragment, as a potential molecular adjuvant, fused with PRV antigen might be a promising and efficient PRV vaccine candidate.

1. Introduction

Pseudorabies (PR) (also called Aujeszky's disease, AD) is an economically important disease of swine and other animals, which is caused by pseudorabies virus (PRV) or suid herpesvirus 1 (SuHV-1) in the worldwide (Lei et al., 2016). The emergence of more virulent PRV strains causes this disease development to spread nearly globally (Muller et al., 2011). The PRV can infect numerous other mammals, such as ruminants, rodents and carnivores with nearly 100% mortality (Freuling et al., 2017; Pensaert and Kluge, 1989). Currently, some means are used to control outbreaks of PR, including immunization with active subunit vaccine and inactivated vaccines. Although attenuated vaccines can generally induce long-lasting immunity, a risk of insufficient attenuation and genetic instability is existent. In addition, inactivated vaccines are less efficient than attenuated vaccines and require repeat doses (Han et al., 2008). Therefore, it is interesting to explore the use of subunit vaccine expressing individual PRV genes, which may provide a safe alternative to the use of attenuated live vaccine strains.

PRV is a member of the family Herpesviridae, subfamily Alphaherpesvirinae (Nauwynck et al., 2007). There are at least 11 different glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, or gN) of PRV that so far have been identified (Dong et al., 2014). Among the different glycoproteins of PRV, gB, gC, and gD are the most important for the development of the antiviral humoral and cellular immune response (Mettenleiter, 1996; Mukamoto et al., 1991). Interestingly, previous studies have shown that regardless of systemic or mucosal administration, recombinant adenoviruses expressing gB or gD could confer the most effective protection against a lethal viral challenge (Han et al., 2008). Hence, we further investigate the effect of gB and gD proteins of PRV on immune responses.

Mucosal and systemic immune respond to early infection and pathogen spread, which is useful for protecting against infectious agents entering the body at mucosal surfaces (Holmgren and Czerkinsky, 2005; McGhee et al., 1992; Neutra and Kozlowski, 2006). The close association between mucosal epithelial cells and the immune effector cells within the laminar propria (Gallichan and Rosenthal, 1998; Holmgren and Czerkinsky, 2005) indicates that delivery of immunogens via the mucosal surface causes mucosal and potential systemic immunity. Fc receptor (FcRn) is reported to transport IgG antibody by the placental or intestinal route in fetuses or newborns life (Ghetie and Ward, 2000; He et al., 2008). Nevertheless, the receptor allows adult to harvest IgG antibody through mucosal surfaces (Baker et al., 2009; Dickinson et al., 1999; Roopenian and Akilesh, 2007), conferring resistance to intestinal

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pathogens (Yoshida et al., 2006). The observation of FcRn transporting IgG across mucosal epithelia suggests that a fusion of antigen to IgG Fc might enable FcRn-mediated transport of the antigen across the mucosal barrier.

In this study, mice were used to explore the immuno protective capacity of potential vaccines against lethal PRV challenge infection as well as to analyze the induced PRV-specific immune response. We worked with the model pathogen PRV, which was primarily initiated infection at the mucosa of the respiratory. Four recombinant baculoviruses were constructed to separately express the glycoproteins gD, gD-IgG2aFc, gB and gB-IgG2aFc. We further tested safety of these recombinants in a PRV mouse challenge model. Taken together, this study may provide an efficient approach for the development of protective mucosal vaccines.

2. Materials and methods

2.1. Viruses and cells

PRV strain GX was isolated from the brain of a diseased newborn piglet, which received PR vaccination in Guangxi province, China in 2018. Porcine kidney PK-15 cells were cultured in DMEM supplemented with 2.5% foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). The PK-15 cells were infected with PRV at a multiplicity of infection (MOI) of 0.01, and then incubated for 1 h in a humidified CO_2 incubator at 37 °C. Sf9 insect cells were used to propagate recombinant baculoviruses and then maintained in Grace's insect medium complemented with 2% FBS at 27 °C.

2.2. Expression of PRV gB, gD, gB-IgG2aFc and gD-IgG2aFc fusion proteins in recombinant baculovirus

The pFBD transfer plasmid expressing PRV glycoproteins was constructed using gateway cloning technique. These plasmids, without the endogenous gD transmembrane region, contained honeybee melittin (HBM) signal peptide that replaced the endogenous gD signal peptide. The GP67 signal sequence was engineered at the N-terminus of gB protein to facilitate secretion during protein production (Fig. 1A). The Fc-fragment of mouse IgG2a containing the hinge, an extended CH2 and a CH3 domain was synthesized. Then, a PCR-based gene assembly approach by mixing the gD (or gB) and the Fc fragment was performed to generate fusions (Fig. 1A and B). The fusions were verified by DNA sequencing. Afterwards, a baculovirus transfer vector designated as pFBD expressing two genes from separate polyhedron promoters was constructed following the method described by Pushko (Sun et al., 2013). Recombinant baculovirus Ac-2gD, Ac-2gD-IgG2aFc, Ac-2gB and Ac-2gB-IgG2aFc were subsequently generated using the Bac-to-Bac system according to the manufacturer's instructions (Fig. 1C).

2.3. Detection and purification of PRV gB, gD, gB-IgG2aFc and gD-IgG2aFc soluble proteins

Sf9 cells were infected with the recombinant baculovirus (Ac-2gD, Ac-2gD-IgG2aFc, Ac-2gB and Ac-2gB-IgG2aFc) at a multiplicity of infection (MOI) of 1, and cultured at 27 °C for 60 h. Then, the proteins from culture supernatant of these cells were extracted and analyzed by western blot analysis. The samples were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride membranes. The membranes were blocked in blocking buffer containing 5% skimmed milk in Tris-buffered saline and Tween 20 (TBS-T) at 4 °C overnight, and incubated with the anti-His monoclonal antibody (1:5000, MBL, Japan) for 2 h at room temperature. The HRP-labelled goat anti-mouse antibody (ABclonal, China) was used as the secondary antibody to incubate the membrane for 1 h at room temperature. After washing thrice with TBS-T, signals were visualized using the ECL chemiluminescence system. Protein bands were analyzed via Image Lab software 4.0.1.

To purify the PRV gD, gD-IgG2aFc, gB and gB-IgG2aFc proteins, we infected Sf9 cells with recombinant baculovirus Ac-2gD, Ac-2gD-IgG2aFc, Ac-2gB and Ac-2gB-IgG2aFc and at a MOI of 0.1. After infection for 120 h, the culture supernatants were centrifuged at 12000 rpm for 30 min and filtered using 0.22 µm filters to remove residual cell debris. PRV gD, gD-IgG2aFc, gB and gB-IgG2aFc proteins were purified by AKTA protein purification apparatus through a Nickel Affinity Chromatography Column (GE, USA) in accordance with the manufacturer's instructions. Then, the purified PRV gD, gD-IgG2aFc, gB and gB-IgG2aFc proteins were confirmed by 12% SDS-PAGE and quantified using a BCA Protein Assay Kit.

2.4. Vaccination and challenge of mice

The 6-week-old female Balb/c mice weighing 20–25 g were purchased from the Laboratory Animal Research Center of Huazhong Agricultural University (Wuhan, China). They were randomly divided into six groups (n = 12/group). The mice were intranasally immunized with 20 µg of gD (Group A), gD-IgG2aFc (Group B), gB (Group C) or gB-IgG2aFc (Group D) protein. The mice in group E were inoculated intramuscularly in the hind legs with 100 µL of 1×10^5 TCID50/ml of PRV ΔgE/ΔgI (Keqian Bio Co.,Ltd, Wuhan, China). The proteins were emulsified with IMS 1313VG adjuvant (Seppic, France), which was showed in previous studies (Zhang et al., 2018), at a ratio of 1:1 (w/w) in accordance with the manufacturer's instructions. Mock-immunized mice (Group F) were given intranasally with PBS. Mice were kept on their backs under anesthesia to allow the inoculum to be taken up. Booster immunization was administered with the same dose at 2 weeks post-vaccination, and the immunized mice were intranasally infected with the virulent PRV GX strain (100 LD50) at four weeks after the final immunization. The challenged mice were examined daily to quantify the number of dead animals. The survival status was calculated. All experimental protocols were approved by the Guide for Laboratory Animal Care and Use and Research Ethics Committee of College of Veterinary Medicine, Huazhong Agricultural University, Hubei, China.

2.5. PRV-specific antibodies measurement

Indirect enzyme-linked immunosorbent assay (ELISA) was used to determine serum PRV-specific antibodies (IgG, IgG1, IgG2a, and IgA) and mucosal PRV-specific antibodies (IgG and IgA) endpoint titers as described previously (Quan et al., 2008; Ye et al., 2011). Briefly, the 96-well flat bottom microtiter plates were coated with 0.5 µg per well of purified gB or gD protein in coating buffer (pH 9.5) at 4 °C overnight. The plate was blocked with 1% bovine serum albumin at 37 °C for 1 h. Then, the serially diluted serum and mucosal samples were added and incubated for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000), IgG1 (1:5000) and IgG2a (1:5000) (ABclonal, USA) were used to measure the Serum PRV-specific antibodies (IgG, IgG1, IgG2a, and IgA) and mucosal PRV-specific antibodies (IgG and IgA) titers at 450 nm. The antibody endpoint titer was based on the highest dilution which gave an OD450 twice that of the naïve group without dilution. Then, the dilution of serum or nasal or bronchial wash fluid was showed in method section.

2.6. Lymphocyte proliferation assay

Lymphocyte proliferation were analyzed using MTS assay kit (Promega, WI, United States) as previously described (Sun et al., 2007; Wang et al., 2013). Briefly, Splenic lymphocytes were isolated from immunized mice using the lymphocyte isolation reagent (TBD, China). Lymphocytes (4×10^6 cells/ml) were seeded into a 96-well plate and grown with 100 µl Roswell Park Memorial Institute (RPMI) 1640 containing 10% FBS. These lymphocytes were stimulated with a mixing

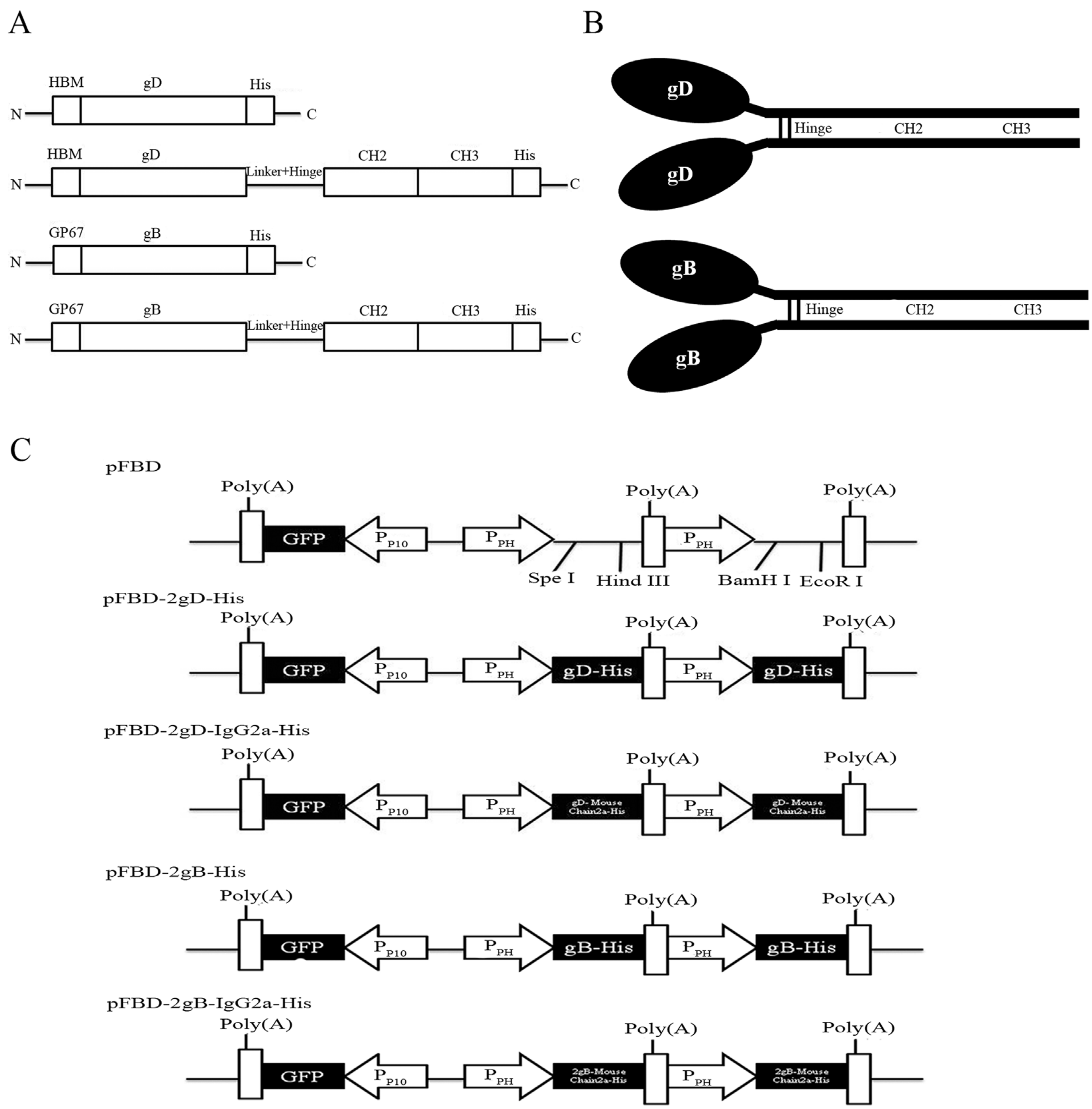

Fig. 1. Schematic representation of the recombinant protein constructs.
(A) Schematic representation of the genetic fusion of HBM, GP67, gD, gB, Linker + Hinge + CH2 + CH3 and His-tag to create HBM-gD-His, HBM-gD-Linker + Hinge + CH2 + CH3-His, GP67-gB-His and GP67-gB-Linker + Hinge + CH2 + CH3-His fusion protein. (B) Schematic illustration of the Fc-mediated dimeric form of gD-Fc and gB-Fc fusion protein. The fourteen codons for glycine and serine residues (GSGGGGSGGGGSGS) were inserted between the gD and Fc fragments. (C) Schematic representation of the recombinant baculoviruses. The eGFP expression cassette was driven by the p10 promoter (P_{P10}). HBM, honeybee melittin. P_{PH}, the polyhedrin promoter of baculovirus. poly (A), polyadenylation signal.

solution of concanavalin A (10 μg/ml), purified gB or gD protein (10 μg/ml) and 100 μl RPMI 1640 containing 10% FBS. After cultivation for 72 h at 37 °C, 20 μl MTS (5 mg/ml in PBS) was added to each well. Following incubation for 4 h at 37 °C, stimulation index (SI) was calculated in accordance with the formula: SI = (OD values of immunized groups – OD values of blank control)/(OD values of negative control group – OD values of blank control).

2.7. Cytokine detection

Splenic lymphocytes were isolated and resuspended in RPMI 1640 medium supplemented with 10% FBS. Cells (4×10^6 cells/ml) were seeded in a 24-well flat-bottom tissue culture plate incubated with 10 μg/ml purified gB or gD protein. After incubation for 48 h at 37 °C, interferon (IFN)-γ and interleukin (IL)-4 levels were detected using corresponding ELISA kits (Neo Bio science, China). the cell-free supernatant was removed and detected using commercially available mouse interferon (IFN)-γ and interleukin (IL)-4 ELISA kit in accordance with the manufacturer's protocol(Neo Bio science, China). The concentrations of different cytokines were determined by the standard curve.

2.8. Serum neutralisation test

Serum neutralisation test was performed to detect PRV antibodies. The serum samples were diluted using DMEM and then added into in 96-well flat-bottomed tissue culture plates (Nunc, USA). A viral suspension with a titre of 200 TCID50 PRV GX strain in 50 μL was added to each well. Following incubation for 1 h at 37 °C, each well was supplemented with 50 μL of the PK-15 cell suspension. After 3 days, the titres of PRV specific neutralisation antibodies were calculated, and expressed as the reciprocal of the highest dilution when PK-15 cell infection was inhibited.

2.9. Necropsy and observation of pathological lesions

All the mice were humanely euthanized by injecting an overdose of intraperitoneal sodium pentobarbital and necropsied on 14 dpc. Brain, Cerebellum and lungs were collected and fixed in 10% neutral-buffered formalin. Paraffin-embedded tissue samples were cut into 4-μm thick slices. After deparaffinization and rehydration, the slices were stained with hematoxylin and eosin (HE). The images were obseved under a light microscope.

2.10. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Student's t-test was used to compare means between two groups. One-way ANOVA followed by Tukey's test was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Expression of recombinant proteins

To confirm the expression of PRV gD, gD-IgG2aFc, gB or gB-IgG2aFc, the protein (gD, gD-IgG2aFc, gB or gB-IgG2aFc) from culture supernatant of sf9 cells infected with recombinant baculovirus was analyzed through western blot analysis. Results showed that in Sf9 cells infected with recombinant baculovirus Ac-gD, Ac-gD-IgG2aFc, Ac-gB or Ac-gB-IgG2aFc, bands at 41, 71, 90 or 120 kDa were detected with anti-His monoclonal antibodies, respectively (Fig. 2A, D, 2G, 2J). Furthermore, the bands at 41, 71, 90 or 120 kDa were also testified with anti-PRV (gD or gB) polyclonal antibodies (Fig. 2B, E, 2H, 2K). By contrast, no specific protein was produced in normal cells (negative control). Then, the PRV gD, gD-IgG2aFc, gB or gB-IgG2aFc protein from Sf9 cells was purified by Ni-NTA chromatography and recombinant PRV protein from culture supernatant of Sf9 cells was verified using 12% SDS-PAGE (Fig. 2C, F, 2I, 2L). In all, these results indicated that PRV gD, gD-IgG2aFc, gB or gB-IgG2aFc protein was confirmed and purified.

3.2. The effect of IgG Fc fusion proteins on mucosal immune

We collected bronchial alveolar lavage (BAL) and nasal washings 10 days after the booster immunization, and detected both for PRV (gD or gB)-specific IgG and IgA levels by ELISA. The IgA level from the gD-IgG2aFc and gB-IgG2aFc immunized groups was significantly higher than that from the gD- and gB immunized groups in the BAL and nasal washings. However, IgA level from live attenuated vaccines immunized group was not detected (Fig. 3A). Moreover, IgG level was also higher in gD-IgG2aFc and gB-IgG2aFc immunized groups, whereas it was dramatically lower in live attenuated vaccines immunized group than in gB-IgG2aFc immunized group (Fig. 3B). Besides, in negative control, mice intranasally immunized with PBS did not show detectable PRV (gD or gB)-specific IgA and IgG antibodies (Fig. 3A and B). Together, these findings revealed that fusion of an antigen to IgG Fc fragment could induce stronger mucosal immune response.

3.3. The effect of IgG Fc fusion proteins on humoral immune responses

For determining the humoral immune response elicited by IgG Fc fusion proteins, serum antibodies of IgG and IgG isotypes (IgG1 and IgG2a) were evaluated by ELISA. All groups, except for the PBS group, produced PRV-specific IgG antibodies at 14, 28 and 42 dpi. Furthermore, the IgG antibody titers from the mice vaccinated with gD-IgG2aFc or gB-IgG2aFc were higher than those from the mice vaccinated with gD or gB (Fig. 4A). In addition, at 28 dpi, the measurement of IgG isotype titers (IgG1 and IgG2a) showed that the mice immunized with IgG Fc fusion proteins induced the high expression level of IgG1, which was a indicator of Th2-type immune response (Fig. 4B). Apparently, the gD-IgG2aFc or gB-IgG2aFc immunized groups produced significantly higher amounts of the IgG2a than gD or gB immunized groups (Fig. 4C). However, for IgG and IgG isotypes (IgG1 and IgG2a) level detection, no difference was observed between gB-IgG2aFc immunized group and live attenuated vaccine immunized group (Fig. 4A, B, 4C). In brief, these results suggested that fusion of an antigen to IgG Fc might effectively promote humoral immune responses.

3.4. The effect of IgG Fc fusion proteins on cellular immune responses

To further investigate the effect of IgG Fc fusion proteins on cellular immune responses, the lymphocyte proliferation was firstly examined. At 42 dpi, the significant differences in SI were observed between the vaccinated groups and the PBS group. Additionally, the gD-IgG2aFc or gB-IgG2aFc groups showed significantly higher SI than gD or gB group. However, there was no effect on the SI from live attenuated vaccine immunized mice and gB-IgG2aFc immunized mice (Fig. 5A). Next, ELISA kit was used to detect intracellular IL-4 and IFN-γ cytokines, which were involved in Th2-biased and Th1 cellular response respectively. Results revealed that IL-4 and IFN-γ secretions were increased in all vaccine inoculated groups compared with PBS group at 42 dpi. Moreover, IL-4 and IFN-γ concentrations in gD-IgG2aFc or gB-IgG2aFc group were significantly higher than those in gD or gB group. We also discovered that IL-4 and IFN-γ concentrations caused by live attenuated vaccines were consistent with that caused by gB-IgG2aFc subunit vaccine (Fig. 5B and C). In all, these data indicated that IgG Fc fusion proteins significantly promoted cellular immune responses.

3.5. The effect of IgG Fc fusion proteins on serum antibody titers

We further analyzed antibody levels induced by vaccination, which were crucial to assess the effects of vaccines. As shown in Fig. 6, no neutralisation activity against the PRV-GX strain was detected in the PBS group throughout the experiment. In contrast, all vaccine inoculated groups showed PRV-specific neutralizing antibody responses at 14, 28 and 42 dpi. The antibody titers in gD-IgG2aFc or gB-IgG2aFc group were significantly higher than those in gD or gB group at 28 and 42 dpi. Moreover, live attenuated vaccines elevated neutralizing antibody titre compared with gB-IgG2aFc at 14 dpi, whereas there was no effect at 28 and 42 dpi. Taken together, these findings suggested that the recombinant protein gD-IgG2aFc or gB-IgG2aFc had good immunogenicity and effectively promoted the antibody titers.

3.6. Protective effects of IgG Fc fusion PRV subunit vaccines

In order to evaluate the protective efficacy of immunity conferred by IgG Fc fusion PRV subunit vaccines, all groups were intranasally challenged with 100 LD50 of the virulent PRV GX strain. In the monitoring period, the survival rates of mice (n = 6/group) were illustrated in Fig. 7. The mice immunized with gD-IgG2aFc or gB-IgG2aFc subunit vaccine showed 83.3% or 100% survival, whereas the mice immunized with gD or gB subunit vaccine had 50% or 66.7% survival. These results indicated that the immunized groups showed a better resistance to PRV infection. Moreover, the live attenuated vaccines provided consistent protective immunity with gB-IgG2aFc-immunized group (100% survival). Additionally, as shown Fig. 8A–C, the histopathology examination results indicated that in mice immunized with gD, gB, gD-IgG2aFc and live attenuated vaccines, there was obvious perivascular microglia hyperplasia in the brains, purkinje neurons injury in the cerebellums and cell necrosis in the livers, whereas, no histopathological lesions

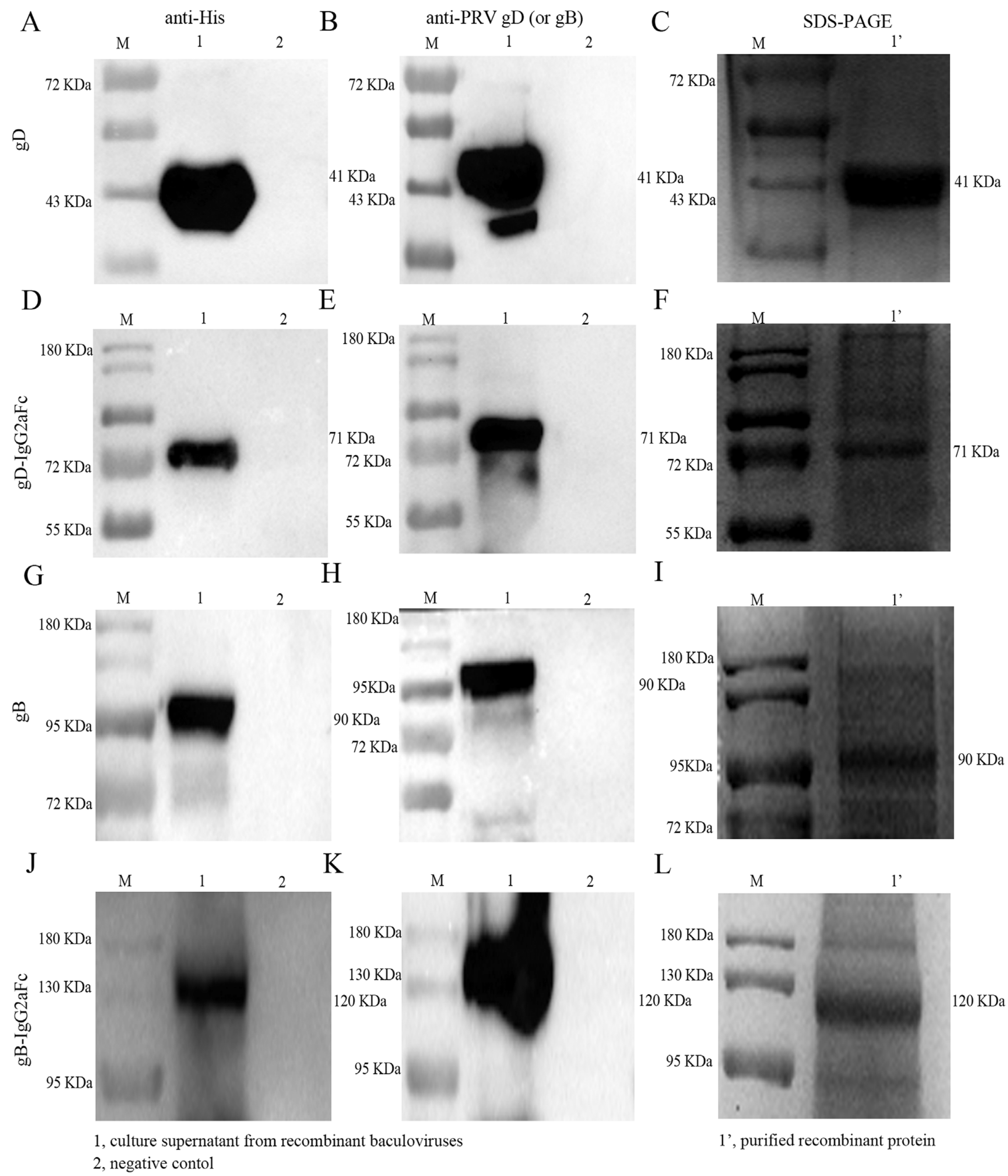

Fig. 2. Detection and purification of PRV gD, gD-IgG2aFc, gB and gB-IgG2aFc proteins.
(A) PRV gD, (D) gD-IgG2aFc, (G) PRV gB and (J) gB-IgG2aFc proteins from culture supernatant of sf9 cells were detected using western blot analysis. An anti-His monoclonal antibody was used as the primary antibody and HRP-labelled goat anti-mouse antibodies were used as the secondary antibody. (B) PRV gD, (E) gD-IgG2aFc, (H) PRV gB and (K) gB-IgG2aFc proteins from culture supernatant of sf9 cells were measured via western blot analysis. An anti-PRV gD (or gB) polyclonal antibody was used as the primary antibody and HRP-labelled goat anti-mouse antibodies were used as the secondary antibody. Lane 1, culture supernatant of sf9 cells infected with recombinant baculoviruses. Lane 2, negative control: culture supernatant of sf9 cells infected without recombinant baculoviruses. (C) The purified PRV gD, (F) gD-IgG2aFc, (I) PRV gB and (L) gB-IgG2aFc proteins were examined through SDS-PAGE. Lane 1′, purified recombinant proteins eluted with five column volumes of 80 mM imidazole.

were observed in mice immunized with gB-IgG2aFc. In conclusion, these data demonstrated that the PRV gB-IgG2aFc subunit vaccine was efficient for PRV infection in mice.

4. Discussion

In current study, we successfully constructed recombinant baculoviruses-expressing two copies of the PRV gD, gD-IgG2aFc, gB and gB-IgG2aFc gene. PRV glycoproteins were efficiently expressed in culture supernatant of sf9 cells. Further experiments revealed the effect of fusion of an antigen to IgG Fc fragment on mucosal immune response, humoral immune responses and cellular immune responses. In addition, we demonstrated that efficacy of IgG Fc fusion PRV subunit vaccines was better than that of live attenuated vaccines.

Accumulating evidence has shown that PRV glycoproteins gD and gB represented major targets for anti-PRV humoral and cellular immune responses (Mettenleiter, 1996; Mukamoto et al., 1991). Moreover, the mouse IgG2a Fc fragment, but not IgG1, could be capable of binding

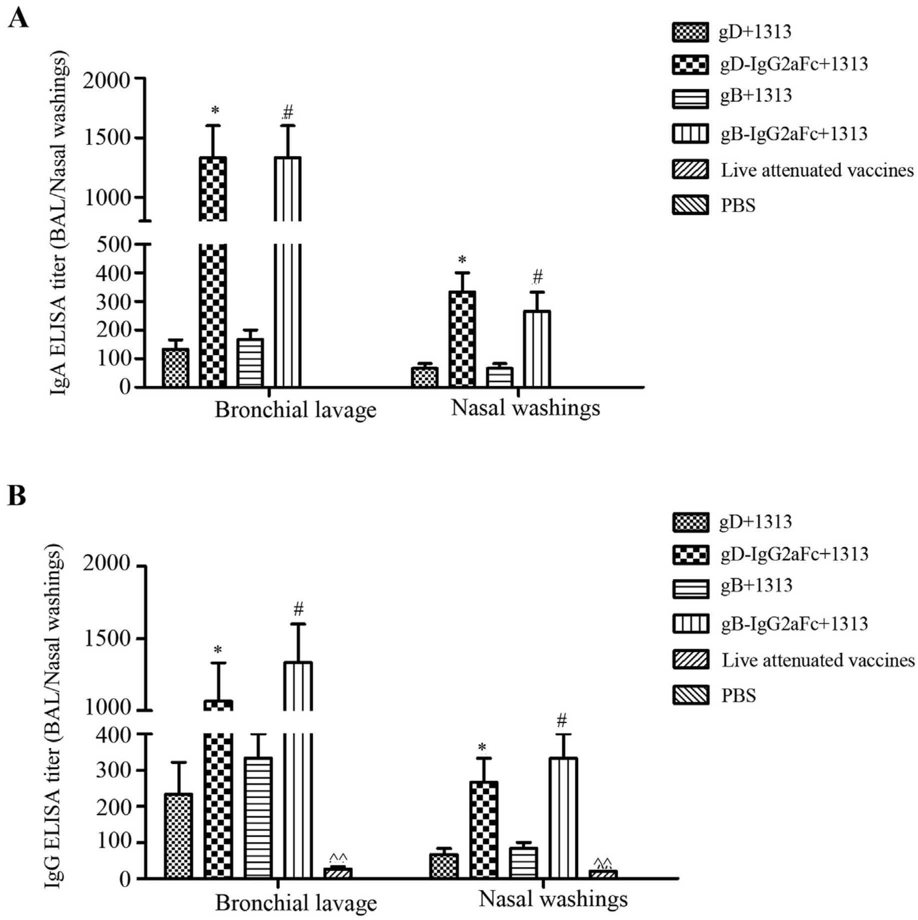

Fig. 3. PRV-specific mucosal immune response detection.

The bronchial lavage (left panel) and nasal washings (right panel) were obtained from mice on the 10 days after the booster immunization, (A) gD (or gB)-specific IgA and (B) IgG titers were determined by ELISA. n = 3. *, $p < 0.05$. #, $p < 0.05$. ^^, $p < 0.01$. Note, * was used to the PRV gD and gD-IgG2aFc groups, # was used to the PRV gB and gB-IgG2aFc groups.1313, whereas ^ was used to the PRV gB-IgG2aFc groups and live attenuated vaccunes.1313 IMS 1313VG adjuvant.

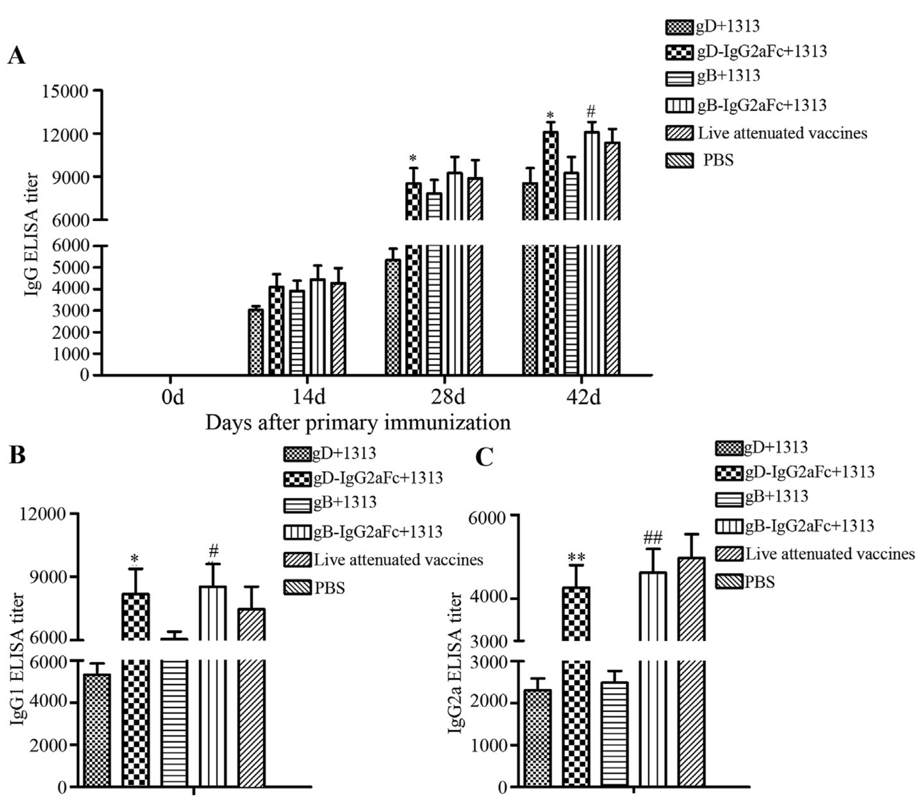

Fig. 4. Humoral immune responses measurement in immunized mice.

(A) The gD (or gB)-specific IgG titer from serum samples was determined by indirect ELISA at 14, 28 and 42 dpi. (B) PRV-specific IgG1 and (C) IgG2a titers were detected at 28 dpi after the final immunization through ELISA. n = 3. *, $p < 0.05$. **, $p < 0.01$. #, $p < 0.05$. ##, $p < 0.01$. Note, * was used to the PRV gD and gD-IgG2aFc groups, whereas # was used to the PRV gB and gB-IgG2aFc groups. dpi, days after primary immunization. 1313, IMS 1313VG adjuvant.

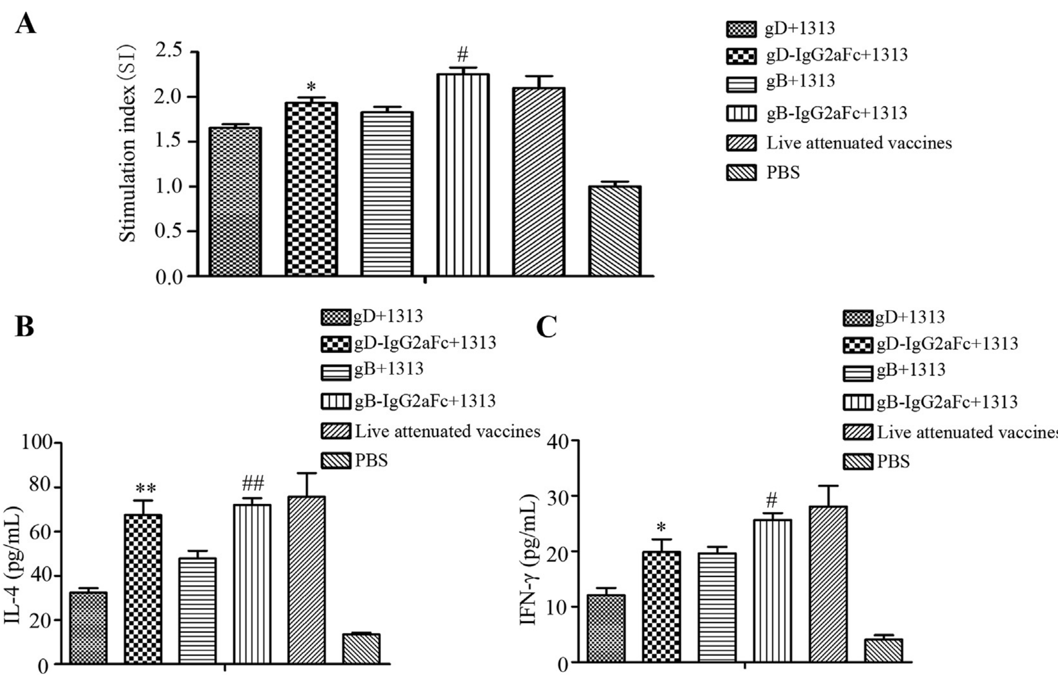

Fig. 5. Cellular immune responses analysis in immunized mice.
(A) Lymphocyte proliferation was detected by the CellTiter 96® Aqueous One Solution Cell Proliferation Assay at 42 dpi. (B) Cytokine IFN-γ and (C) IL-4 levels in the supernatant of the stimulated T cells were determined through cytokine ELISA at 42 dpi. n = 3. *, $p < 0.05$. **, $p < 0.01$. #, $p < 0.05$, ##, $p < 0.01$. Note, * was used to the PRV gD and gD-IgG2aFc groups, whereas # was used to the PRV gB and gB-IgG2aFc groups. dpi, days after primary immunization. 1313, IMS 1313VG adjuvant.

mouse FcγRI, a high-affinity receptor for IgG (Ye et al., 2011). Interestingly, in our study, we also concentrated on PRV glycoproteins gD and gB. The gD and gB protective antigen genes were linked to the mouse IgG2aFc gene to generate the recombinant baculoviruse expression system of gD-IgG2aFc and gB-IgG2aFc, suggesting that a modified form of the mouse IgG2aFc fragment facilitating the IgG Fc fusion PRV subunit vaccines is established.

Earlier evidences have revealed that antibody-mediated feedback was that antibodies concurrently administered with an antigen could regulate the immune response of antigens (Brady, 2005). It is reported that Fc region showed potent immune effector functions via engaging FcRs and serum complement proteins (Sang, 2013). Therefore, in our study, to evaluate the function of IgG Fc fusion PRV subunit vaccines in mice, we detected both mucosal and systemic immune responses. For mucosal immune response measurement, our present study demonstrated that gD-IgG2aFc and gB-IgG2aFc induced high level of PRV (gD or gB)-specific IgA and IgG in the BAL and nasal washings. However, live attenuated vaccines did not induced production of IgA, and IgG level was low in live attenuated vaccines immunized group compared with in gB-IgG2aFc immunized group. Notably, previous studies have been proved that traditional PRV vaccines, which had some problems of safety, could result in the spread of PRV in different species to some extent (Li et al., 2017). Therefore, these findings indicate that IgG Fc fusion PRV subunit vaccines might be safe in mice with PRV infection.

Further, humoral or cellular immune responses against PRV infection has been discovered to be induced by recombinant DNA vaccines expressing gC and gD (Monteil et al., 2000; Rajcani et al., 2018; Yoon et al., 2006). Then, the stimulation of humoral immune responses was examined in our study. Reportedly, Zaharatos et al. discovered that HIV-1 and influenza antigens synthetically linked to IgG2a Fc could cause potent humoral responses in mice (Zaharatos et al., 2011). Furthermore, Hong et al. proved that lentivector expressing HBsAg and immunoglobulin Fc fusion antigen could induce humoral immune responses (Hong et al., 2011). These findings suggest that Fc fusion

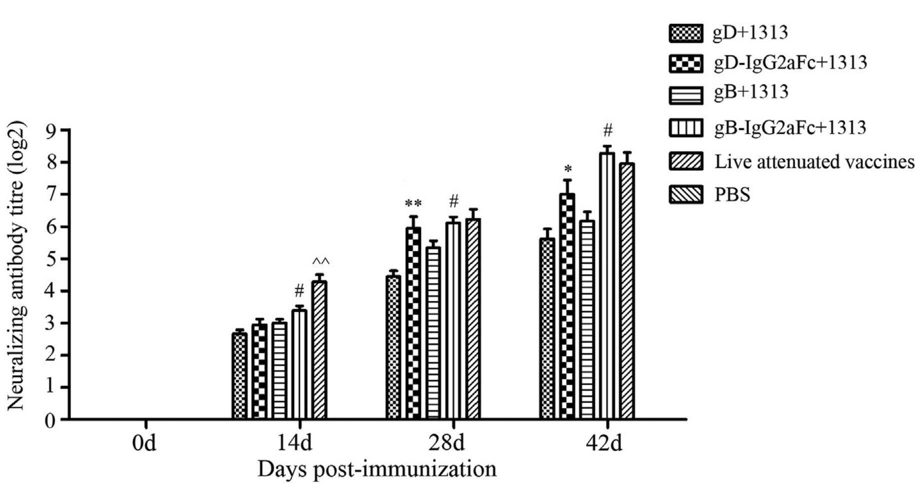

Fig. 6. Neutralizing antibody titers against PRV GX strain.
The neutralizing ability of antisera generated against PRV GX strain were calculated and expressed as the log
2 of the reciprocal of the highest serum dilution when PK-15 cell infection was inhibited. *, $p < 0.05$. **, $p < 0.01$. #, $p < 0.05$. ^^, $p < 0.01$. Note, * was used to the PRV gD and gD-IgG2aFc groups, # was used to the PRV gB and gB-IgG2aFc groups.1313, whereas ^ was used to the PRV gB-IgG2aFc groups and live attenuated vaccunes. 1313, IMS 1313VG adjuvant.

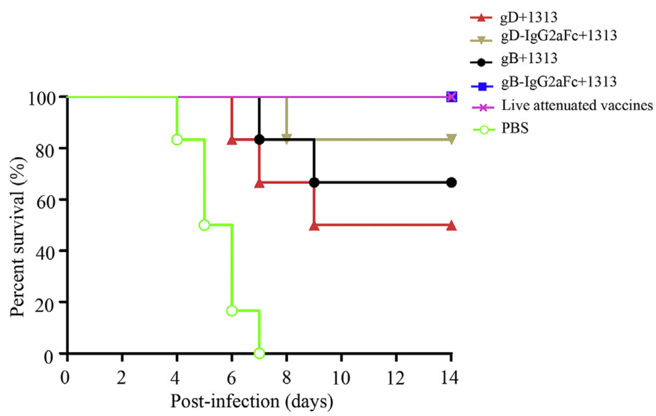

Fig. 7. IgG Fc fusion proteins provided protective immunity to mice intranasally challenge with virulent PRV-GX strain.
Four weeks after the immunization, mice in five groups were intranasally challenged with 100 LD50 of PRV-GX strain. Percentage survival on the indicated days was calculated as: survival rate (%) = (numbers of mice surviving/numbers of total mice) × 100%. 1313, IMS 1313VG adjuvant.

protein might play an important role in humoral immune responses. Interestingly, functional analysis showed that IgG Fc fusion PRV subunit vaccines elevated PRV-specific IgG and IgG isotypes (IgG1 and IgG2a) levels, and increased neutralizing antibody titers at 28, 42 dpi. However, the levels of PRV-specific IgG and IgG isotypes (IgG1 and IgG2a), and neutralizing antibody titers at 28, 42 dpi from live attenuated vaccine immunized mice were consistent with that from gB-IgG2aFc immunized mice. Importantly, at 14 dpi, we found that only PRV gB-IgG2aFc proteins caused the increase of neutralizing antibody titers and live attenuated vaccines enhanced neutralizing antibody titers compared with PRV gB-IgG2aFc, which suggesting that the effect of gB-IgG2aFc subunit vaccine on PRV infection might be the best and live attenuated vaccines might induced neutralizing antibody early. However, there was no significant difference in duration of neutralizing antibodies induced by PRV gB-IgG2aFc and live attenuated vaccines. These results indicate that the Fc fragment fused with PRV antigen can further improve the humoral immune responses.

Previous studies of cell-mediated immunity induced by vaccination of recombinant heterologous vectors, which expressed gB, gC and gD, was assessed in mice or pigs (Katayama et al., 1997; van Rooij et al., 2000; Yoon et al., 2006). Moreover, high level of cell-mediated immunity was useful for preventing complete virus shedding (Zhang et al., 2019). In our study, we confirmed that vaccinated groups showed higher stimulated PRV-specific T-lymphocyte proliferative responses and production of intracellular IL-4 and IFN-γ cytokines than PBS group. Moreover, IgG Fc fusion PRV subunit vaccines dramatically facilitated PRV-specific T-lymphocyte proliferative responses and increased the production of intracellular IL-4 and IFN-γ cytokines compared with non-tagged proteins. Approximately 70 pg/mL of IL-4 was produced by IgG Fc fusion PRV subunit vaccines. IL-4 was found to be important for maintaining Th2 immunity (Finkelman et al., 1991; Kopf et al., 1993; McCoy et al., 2010). However, no difference was shown in SI, IL-4 and IFN-γ from live attenuated vaccine-immunized mice and PRV gB-IgG2aFc-immunized mice. These results suggest that Th2-specific cellular immune responses are induced in the vaccinated groups, and the Fc fragment fused with PRV antigen can show a stronger cellular immune response. Besides, the survival rate of mice immunized with the gB-IgG2aFc proteins and live attenuated vaccines was 100%, and that from mice immunized with the gD-IgG2aFc proteins showed 83.3%. No histopathological lesions were observed in mice immunized with gB-IgG2aFc proteins, whereas the gD-IgG2aFc proteins and live attenuated vaccines exhibited histopathological lesions. Consistent with

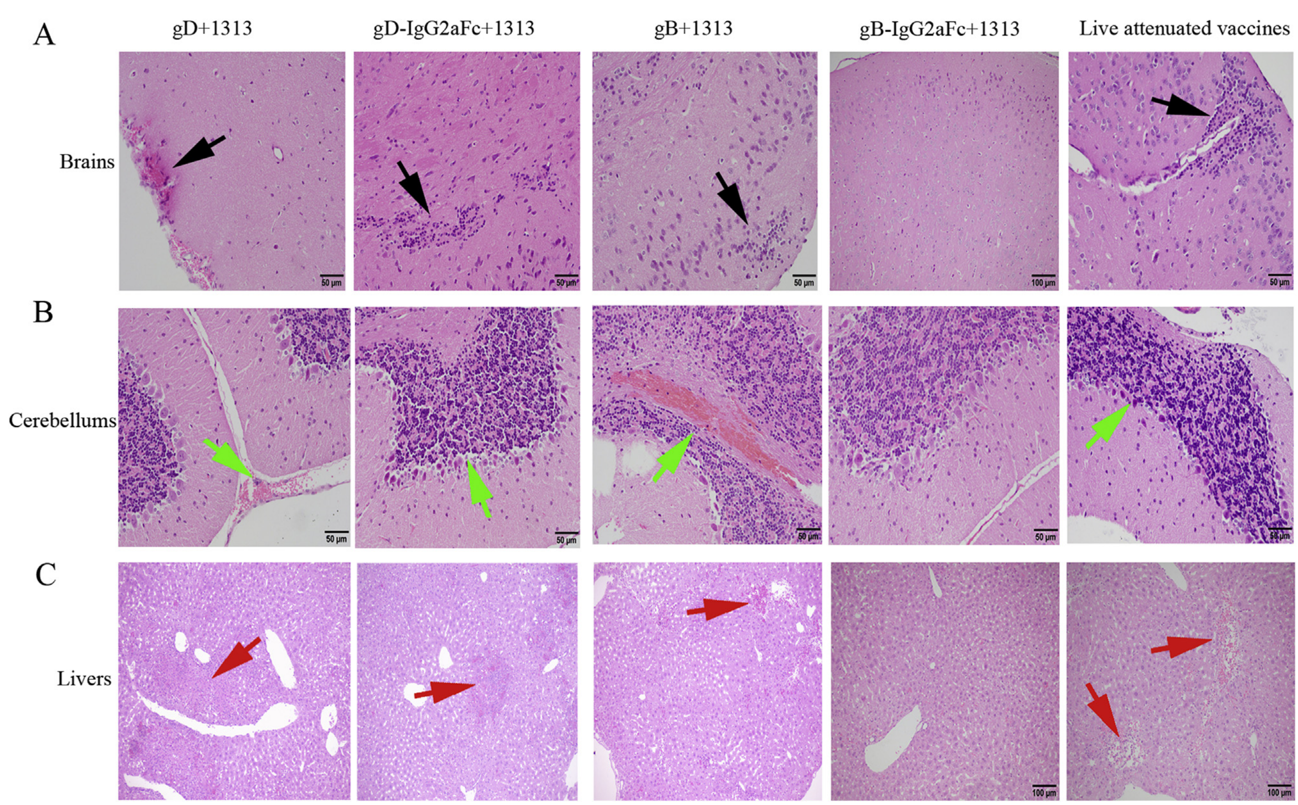

Fig. 8. Histopathology examination.
In immunized mice at 14 d post-challenge, HE staining was used to detect the pathological lesions of (A) brain, (B) cerebellum and (C) liver tissues. Scale bar, 50 μm or 100 μm. Black arrow indicated perivascular microglia hyperplasia in the brains. Green arrow indicated purkinje neurons injury in the cerebellums. Red arrow indicated cell necrosis in the livers. HE, hematoxylin and eosin staining. 1313, IMS 1313VG adjuvant.

that traditional PRV vaccines exhibited some problems of safety (Li et al., 2017). Taken together, these findings indicate that the recombinant baculoviruses-expressing PRV gB subunit vaccine protected mice against PRV infection.

However, the mechanism underlying the function of recombinant PRV subunit vaccine in other animals, such as swine, is still not fully clarified. Therefore, additional studies will be conducted in the future.

In summary, we demonstrated that the recombinant PRV subunit vaccine was effectively generated by the mouse IgGFc fragments fused with PRV antigen, and the fusion of PRV glycoproteins to IgG Fc enhanced protective immunity against PRV. The results suggest that mouse IgG2a Fc fragment can function as a potential molecular adjuvant, and the fusion of PRV glycoproteins to IgG Fc may be a safe and effective subunit vaccine candidate for PRV.

Author contributions

Designed the experiments: P.Q., X.L. and H.C.; Performed the experiments: J.L., GH., HZ. and H.Y.; Analyzed the data: J.L., GH. and HZ.; Wrote the paper: J.L.; Proofed the manuscript: P.Q., X.L. and H.C. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

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References

Baker, K., Qiao, S.W., Kuo, T., Kobayashi, K., Yoshida, M., Lencer, W.I., Blumberg, R.S., 2009. Immune and non-immune functions of the (not so) neonatal Fc receptor. FcRn. Seminars in immunopathology 31, 223–236.

Brady, L.J., 2005. Antibody-mediated immunomodulation: a strategy to improve host responses against microbial antigens. Infect. Immun. 73, 671–678.

Dickinson, B.L., Badizadegan, K., Wu, Z., Ahouse, J.C., Zhu, X., Simister, N.E., Blumberg, R.S., Lencer, W.I., 1999. Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. J.Clin. Invest. 104, 903–911.

Dong, B., Zarlenga, D.S., Ren, X., 2014. An overview of live attenuated recombinant pseudorabies viruses for use as novel vaccines. J Immunol Res 824630, 5.

Finkelman, F.D., Pearce, E.J., Urban Jr., J.F., Sher, A., 1991. Regulation and biological function of helminth-induced cytokine responses. Immunol. today 12, A62–A66.

Freuling, C.M., Muller, T.F., Mettenleiter, T.C., 2017. Vaccines against pseudorabies virus (PrV). Vet. Microbiol. 206, 3–9.

Gallichan, W.S., Rosenthal, K.L., 1998. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. J. Infect. Dis. 177, 1155–1161.

Ghetie, V., Ward, E.S., 2000. Multiple roles for the major histocompatibility complex class I- related receptor FcRn. Annu. Rev. Immunol. 18, 739–766.

Han, Y.W., Aleyas, A.G., George, J.A., Kim, S.J., Kim, H.K., Yoon, H.A., Yoo, D.J., Kang, S.H., Kim, K., Eo, S.K., 2008. Polarization of protective immunity induced by replication-incompetent adenovirus expressing glycoproteins of pseudorabies virus. Exp. Mol. Med. 40, 583–595.

He, W., Ladinsky, M.S., Huey-Tubman, K.E., Jensen, G.J., McIntosh, J.R., Bjorkman, P.J., 2008. FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. Nature 455, 542–546.

Holmgren, J., Czerkinsky, C., 2005. Mucosal immunity and vaccines. Nat. Med. 11, S45–S53.

Hong, Y., Peng, Y., Mi, M., Xiao, H., Munn, D.H., Wang, G.Q., He, Y., 2011. Lentivector expressing HBsAg and immunoglobulin Fc fusion antigen induces potent immune responses and results in seroconversion in HBsAg transgenic mice. Vaccine 29, 3909–3916.

Katayama, S., Okada, N., Yoshiki, K., Okabe, T., Shimizu, Y., 1997. Protective effect of glycoprotein gC-rich antigen against pseudorabies virus. J. Vet. Med. Sci. 59, 657–663.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., Kohler, G., 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362, 245–248.

Lei, J.L., Xia, S.L., Wang, Y., Du, M., Xiang, G.T., Cong, X., Luo, Y., Li, L.F., Zhang, L., Yu, J., Hu, Y., Qiu, H.J., Sun, Y., 2016. Safety and immunogenicity of a gE/gI/TK gene-deleted pseudorabies virus variant expressing the E2 protein of classical swine fever virus in pigs. Immunol. Lett. 174, 63–71.

Li, Y., Yan, S., Li, X., Yang, Q., Guo, L., Wang, Y., Xiao, Y., Tan, F., Tian, K., 2017. From mouse to pig: is PRV vaccine safe across two species? Virus Res. 236, 44–49.

McCoy, M.E., Finkelman, F.D., Straus, D.B., 2010. Th2-specific immunity and function of peripheral T cells is regulated by the p56Lck Src homology 3 domain. J. Immunol. 185, 3285–3294.

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M., Kiyono, H., 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10, 75–88.

Mettenleiter, T.C., 1996. Immunobiology of pseudorabies (Aujeszky's disease). Vet. Immunol. Immunopathol. 54, 221–229.

Monteil, M., Le Pottier, M.F., Ristov, A.A., Cariolet, R., L'Hospitalier, R., Klonjkowski, B., Eloit, M., 2000. Single inoculation of replication-defective adenovirus-vectored vaccines at birth in piglets with maternal antibodies induces high level of antibodies and protection against pseudorabies. Vaccine 18, 1738–1742.

Mukamoto, M., Watanabe, I., Kobayashi, Y., Icatlo Jr., F.C., Ishii, H., Kodama, Y., 1991. Immunogenicity in Aujeszky's disease virus structural glycoprotein gVI (gp50) in swine. Vet. Microbiol. 29, 109–121.

Muller, T., Hahn, E.C., Tottewitz, F., Kramer, M., Klupp, B.G., Mettenleiter, T.C., Freuling, C., 2011. Pseudorabies virus in wild swine: a global perspective. Arch. Virol. 156, 1691–1705.

Nauwynck, H., Glorieux, S., Favoreel, H., Pensaert, M., 2007. Cell biological and molecular characteristics of pseudorabies virus infections in cell cultures and in pigs with emphasis on the respiratory tract. Vet. Res. 38, 229–241.

Neutra, M.R., Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. Nat. Rev. Immunol. 6, 148–158.

Pensaert, M.B., Kluge, P., 1989. Pseudorabies virus (Aujeszky's disease). In: Pensaert, M. (Ed.), Virus Infections of Porcines. Elsevier Science Publishers, New York, pp. 39–64.

Quan, F.S., Compans, R.W., Nguyen, H.H., Kang, S.M., 2008. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J. Virol. 82, 1350–1359.

Rajcani, J., Banati, F., Szenthe, K., Szathmary, S., 2018. The potential of currently unavailable herpes virus vaccines. Expert Rev. Vaccines 17, 239–248.

Roopenian, D.C., Akilesh, S., 2007. FcRn: the neonatal Fc receptor comes of age. Nat. Rev. Immunol. 7, 715–725.

Sang, T.J., 2013. Tailoring immunoglobulin Fc for highly potent and serum-stable therapeutic antibodies. Biotechnol. Bioproc. Eng. 18, 625–636.

Sun, S.Q., Liu, X.T., Guo, H.C., Yin, S.H., Shang, Y.J., Feng, X., Liu, Z.X., Xie, Q.G., 2007. Protective immune responses in Guinea pigs and swine induced by a suicidal DNA vaccine of the capsid gene of swine vesicular disease virus. J. Gen. Virol. 88, 842–848.

Sun, Y., Tian, D.Y., Li, S., Meng, Q.L., Zhao, B.B., Li, Y., Li, D., Ling, L.J., Liao, Y.J., Qiu, H.J., 2013. Comprehensive evaluation of the adenovirus/alphavirus-replicon chimeric vector-based vaccine rAdV-SFV-E2 against classical swine fever. Vaccine 31, 538–544.

van Rooij, E.M., Haagmans, B.L., Glansbeek, H.L., de Visser, Y.E., de Bruin, M.G., Boersma, W., Bianchi, A.T., 2000. A DNA vaccine coding for glycoprotein B of pseudorabies virus induces cell-mediated immunity in pigs and reduces virus excretion early after infection. Vet. Immunol. Immunopathol. 74, 121–136.

Wang, Y.P., Liu, D., Guo, L.J., Tang, Q.H., Wei, Y.W., Wu, H.L., Liu, J.B., Li, S.B., Huang, L.P., Liu, C.M., 2013. Enhanced protective immune response to PCV2 subunit vaccine by co-administration of recombinant porcine IFN-gamma in mice. Vaccine 31, 833–838.

Ye, L., Zeng, R., Bai, Y., Roopenian, D.C., Zhu, X., 2011. Efficient mucosal vaccination mediated by the neonatal Fc receptor. Nat. Biotechnol. 29, 158–163.

Yoon, H.A., Aleyas, A.G., George, J.A., Park, S.O., Han, Y.W., Kang, S.H., Cho, J.G., Eo, S.K., 2006. Differential segregation of protective immunity by encoded antigen in DNA vaccine against pseudorabies virus. Immunol. Cell Biol. 84, 502–511.

Yoshida, M., Kobayashi, K., Kuo, T.T., Bry, L., Glickman, J.N., Claypool, S.M., Kaser, A., Nagaishi, T., Higgins, D.E., Mizoguchi, E., Wakatsuki, Y., Roopenian, D.C., Mizoguchi, A., Lencer, W.I., Blumberg, R.S., 2006. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. J.Clin. Invest. 116, 2142–2151.

Zaharatos, G.J., Yu, J., Pace, C., Song, Y., Vasan, S., Ho, D.D., Huang, Y., 2011. HIV-1 and influenza antigens synthetically linked to IgG2a Fc elicit superior humoral responses compared to unmodified antigens in mice. Vaccine 30, 42–50.

Zhang, C., Liu, Y., Chen, S., Qiao, Y., Guo, M., Zheng, Y., Xu, M., Wang, Z., Hou, J., Wang, J., 2019. A gD&gC-substituted pseudorabies virus vaccine strain provides complete clinical protection and is helpful to prevent virus shedding against challenge by a Chinese pseudorabies variant. BMC Vet. Res. 15, 2.

Zhang, H., Wen, W., Hao, G., Chen, H., Qian, P., Li, X., 2018. A subunit vaccine based on E2 protein of atypical porcine pestivirus induces Th2-type immune response in mice. Viruses 10.