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The composite biological adjuvants enhance immune response of porcine circovirus type2 vaccine

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ABSTRACT

The porcine circovirus (PCV) is one of the most economically important infection diseases of pigs. Granulocyte–macrophage colony-stimulating factor (GM-CSF) or Flic as an immune adjuvant has been shown to enhance the immunogenicity of vaccines in previous study. However, the composite biological adjuvants stimulate more effective immunological response. In this study, the porcine GM-CSF (pGM-CSF) and FliC protein were expressed by pSUMO in *E.coli* Rosetta (DE3) and purified by Ni-NTA Sepharose, respectively. The immunogenicity of PCV vaccine with pGM-CSF and FliC was firstly evaluated to identify the immunoenhancement in mice. The results indicated that mice immunized with vaccine + pGM-CSF + FliC enhanced immune responses ability significantly and quickly. Then, the immune response level of PCV vaccine with pGM-CSF and FliC was assessed in piglets. The results indicated that pigs immunized with vaccine + pGM-CSF + FliC showed significantly higher PCV antibody level than those immunized with vaccine and single adjuvant or vaccine alone. Furthermore, pigs in the vaccine + pGM-CSF + FliC group elicited stronger CD4+ and CD8 + T cells proliferative responses than those in all other groups and showed the effectively up-regulated transcriptional level of IL-1, IL-8 and IL-17 stimulating the immune system. We demonstrated that GM-CSF and FliC as composite biological adjuvants of the vaccine showed a stronger immune response and it is promising application for vaccines to against PCV.

1. Introduction

The porcine circovirus (PCV) is an important viral infection disease in the pig population and is the major pathogen of a series of syndromes collectively referred to as porcine-circovirus-associated disease (PCVAD) (Rose et al., 2012; Chae, 2005). PCVAD is the cause of a major animal-health crisis and causes tremendous economic losses to the swine industry worldwide (Ge et al., 2012). Vaccination is still the main way to protect pigs from PCV, and most vaccines are live attenuated and inactivated vaccines which do not exert potent protective effect against PCV infection (Beach and Meng, 2012; Schijns and Lavelle, 2014). Therefore, it is necessary to exploit effective adjuvants to enhance the immune efficacy of these vaccines.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) activates macrophages, dendritic cells (DCs) and neutrophils, and induces antigen presenting cell to differentiate and maturate, which enhances immune response and protective immunity (Burgess and Metcalf, 1980; Cruciani et al., 2007; Shi et al., 2006). Sasaki Md et al. found that GM-CSF increased the immunogenicity of recombinant HBV vaccine in HBV infected individuals (Sasaki and Foccacia, 2003). GM-CSF has a broad prospect because of its intense immune reaction in vaccine (Slingluff et al., 2003). As an adjuvant, the advantage of GM-CSF is that it enhances immune response rapidly, while its disadvantage is that the action time is short (Aguilar and Rodríguez, 2007; Chen et al., 2014). Toll-like receptors (TLRs) play a critical role in innate sensing of infection and in the initiation of adaptive immune responses (Barton and Medzhitov, 2002). TLR5 is one of the TLRs family members, its ligand is FliC that promotes the release of proinflammatory

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cytokine when it recognizes and binds to TLR5 (Hayashi et al., 2001). Previous study has demonstrated that the genetic fusion product including FliC and the antigen enhanced specific immune response of the antigen (Huleatt et al., 2008), and FliC as an adjuvant induced innate immune response and activate adaptive immune response (Barton and Medzhitov, 2002; Hayashi et al., 2001). As the advantages, FliC protein is effective at low dose and induces long-lasting immunity (Aguilar and Rodríguez, 2007). Therefore, it is a reasonable strategy to apply porcine GM-CSF (pGM-CSF) and FliC as composite biological adjuvants of the PCV vaccines to enhance their immune efficacy.

In this study, pGM-CSF and FliC proteins were expressed and purified, as composite biological adjuvants, and their immune efficacy was evaluated in the mice or piglets injected with inactivated PCV vaccine.

2. Materials and methods

2.1. Cell and viruses

TF-1 cell line was purchased from Peking union medical college cell center and cultured in RPM1640 medium containing 10% FBS and 5 ng·mL^{-1} GM-CSF standard protein (R&D Systems, USA). PCV type2 vaccine inactivated (Strain WH) was purchased from Wuhan Keqian Animal Biological Products Co., Ltd and used for immunization experiment in mice and pigs. NDV Anhinga strain (Clone30) was from our laboratory stock.

2.2. Animals

One-day-old chickens were purchased from Harbin Veterinary Research Institute. Seven-week-old female BABL/C mice with a mean weight of 18–22 g were purchased from the Experimental Animal Center of Chang Chun Yi Si Company and housed individually in cages of standard size. Mice were fed with standard rodent chow and provided water ad libitum. Four-week-old piglets with a mean weight of 15 kg were purchased from a PCV-free farm and each group of piglets was housed in a room separately. All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Institutional Review Board of the Northeast Agricultural University Institute of Biomedicine.

2.3. The preparation of pGM-CSF and FliC protein

2.3.1. Expression and purification of pGM-CSF and FliC protein

pGM-CSF (GenBank: AY116504.1) and FliC sequence (GenBank: JX847136.1) were synthesized by Bio-serve and contained restriction enzyme cutting sites of *Bsa*I and *Hind*III. The synthesized pGM-CSF and FliC sequence were digested by *Bsa*I and *Hind*III and ligated into the expression vector pSUMO, respectively. The two recombinant plasmids were transformed into *E. coli* Rosetta (DE3). The expression of pGM-CSF and FliC protein was analyzed by SDS-PAGE. Then, the two proteins were purified by a Ni Sepharose 6 Fast Flow column in AKTA Purifier (GE Healthcare, USA), respectively. The endotoxin was removed from the purified two proteins by ToxinEraser™ Endotoxin Removal Kit (GenScript, NanJing, China), and the purified two proteins were analyzed by SDS-PAGE.

2.3.2. The activity analysis of pGM-CSF protein

Because of TF-1 cells require GM-CSF for cell division and proliferation (Wang et al., 2005), the biological activity of the purified pGM-CSF protein was measured on TF-1 cells by MTT colorimetric method. TF-1 cells were resuspended in culture medium without GM-CSF standard at 4.0×10^5/mL after washing three times. GM-CSF standard and pGM-CSF protein were diluted to 10 ng·mL^{-1} with the basic culture medium, which were again diluted to various concentrations (8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 ng·mL^{-1}), respectively. In 96-well plate, 50 µL cell culture medium and 50 µL GM-CSF standard or pGM-CSF protein diluent were added in each well in the presence of 5% CO_2 at 37 °C for 52 h. After that, 20 µL MTT solution was added in the presence of 5% CO_2 at 37 °C for 5 h. Absorbance at 570 nm was recorded after adding 100 u l lysate, and the glucose consumption rate was calculated.

2.3.3. Detecting the activity of FliC protein

The activity of the purified FliC protein was examined by immunizing chickens with NDV vaccine (clone30). One-day-old chickens were randomly divided into three groups (n = 10 chickens per group), including clone30 + 10 µg FliC, clone30 and normal (PBS). The blood of each chicken was collected in chicken wing vein before the immunization, and serum was separated from whole blood to test the titers of NDV maternal antibody by hemagglutination-inhibition (HI). The experiment groups (clone30 + 10 µg FliC or clone30) were immunized with 10^6 $TCID_{50}$ dose of clone30 by nasal drip or eye droppings, subsequently, each chicken was injected 10 µL FliC by intramuscular injection in clone30 + 10 µg FliC group. The control group was administrated with PBS solution by nasal drip or eye droppings. After immunization, the serum of each chicken was collected to test NDV antibody titers by HI test at 7, 14 and 21 days.

2.4. The immune effect of pGM-CSF and FliC protein in mice

2.4.1. Animal immunization

Seven-week-old female BABL/C mice were divided into six groups with 15 mice per group. The experiment groups were inoculated intramuscularly 50 µg pGM-CSF + PCV vaccine, 25 µg pGM-CSF + PCV vaccine, 12 µg pGM-CSF + PCV vaccine, 10 µg FliC + PCV vaccine, 25 µg pGM-CSF + 10 µg FliC + PCV vaccine and PCV vaccine alone, respectively. Mice were inoculated intramuscularly with PBS as control group.

2.4.2. Detection of PCV-specific antibody by ELISA

The blood samples were collected and isolated serum from three mice each group at 2, 4, 6, 8 and 10 days after immunization. PCV-specific antibody was detected by ELISA. 96-well plate was covered with 100 µL purified PCV antigen (10 µg mL^{-1}) at 4 °C overnight. Triplicate samples were washed three times with PBST and blocked with 5% skimmed milk (200 µL/well) in PBS at 37 °C for 3 h. After washing, each well was incubated with serum samples (1:50) at 37 °C for 1 h. After washing, the HRP-goat anti-mouse antibody (1:7 500 Sigma, USA) was incubated at 37 °C for 1 h. The test was developed by using TMB solution (100 µL/well) at 37 °C for 15 min and the reaction was terminated by the 2 mol/L H_2SO_4 (50 µL/well). The absorbance of each well was measured by the microplate reader at 450 nm. Three negative controls were included: control1 (without serum), control2 (without HRP-goat anti-mouse antibody), and control3 (without PCV antigen).

2.4.3. Flow cytometric (FCM) analysis

The blood samples were collected from three mice each group at 2, 4, 6, 8 and 10 days after immunization in tubes containing EDTA anticoagulant. Red blood cell lysis buffer (Beyotime, Beijing, China) was added into the tubes in order to get white blood cells, which were harvested and resuspended in PBS and seeded with tubes at a final concentration of 10^6 cells. Cells were incubated on ice with the FITC-labeled anti-mouse CD3e antibody (Miltenyi Biotec, Germany), PE-labeled anti-mouse CD4 antibody (Miltenyi Biotec, Germany), APC-labeled anti-mouse CD8a antibody (Miltenyi Biotec, Germany) at saturating concentration for 45 min. The stained cells were washed twice with 1 ml PBS and resuspended in 500 µL PBS for being detected by FCM (BD FACSAria™ IIu Cell Sorter, BD Biosciences, USA).

2.5. The immune effect of pGM-CSF and FliC proteins in piglets

2.5.1. Animal immunization

Four-week-old piglets were randomly assigned to four groups with three pigs each group. Pigs in group 1 (negative control group) were inoculated with 2 ml PBS intramuscularly. Pigs in group 2 were inoculated with 2 ml PCV vaccine intramuscularly. Pigs in group 3 were inoculated with 2 ml PCV vaccine and pGM-CSF (1.25 mg kg^{-1}) intramuscularly. Pigs in group 4 were inoculated with 2 ml PCV vaccine, pGM-CSF (1.25 mg kg^{-1}) and FliC (0.5 mg kg^{-1}) intramuscularly.

2.5.2. Detection of PCV-specific antibody by ELISA

The blood samples were collected at 2, 4, 6, 8, 10 and 13 days after immunization and the serum was separated to measure PCV-specific antibody by ELISA. Briefly, 96-well plate was covered with 100 μL purified PCV antigen (10 μg mL^{-1}) at 4 °C overnight and blocked (200 μL/well) with 5% skimmed milk in PBS at 37 °C for 3 h. After washing, each well was incubated with serum samples (1:50) at 37 °C for 1 h. After washing, the HRP-rabbit anti-pig antibody (1:7 500 Sigma, USA) was incubated at 37 °C for 1 h. The absorbance of samples was measured by the microplate reader at 450 nm.

2.5.3. FCM analysis

The blood samples were collected in EDTA anticoagulant tubes at 2, 4, 6, 8, and 10 days after immunization. White blood cells were isolated from the whole blood by red blood cell lysis buffer and incubated with PE/Cy5-labeled anti-pig CD3, FITC-labeled anti-pig CD4 and PE-labeled anti-pig CD8 (Abcam, USA). After 1 h, cells were washed twice with PBS to remove unbound antibodies. Subsequently, cells were analyzed by FCM.

2.5.4. Analysis of cytokine by real-time PCR

White blood cells were collected at 2, 4, 6, 8, and 10 days after immunization. Total RNA was isolated from the white blood cells using Trizol reagent (Invitrogen). cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA). Target cDNA was analyzed by real-time PCR with the iTaq SYBR Green Supermix by an ABI 7500 system (Applied Biosystems, Foster City, CA) according to the manufacturers protocols. The following primers were used (sequence 5′-3′):

IL-1: (Forward: GTGATGGCTAACTACGG; Reverse: GTCTGCCT GATGCTCTT); IL-8: (Forward: TTGGCTGTTGCCTTCTTG; Reverse: GGTGGAATGCGTATTTATG); IL-17: (Forward: TCGTG AAGGCGGGA ATC; Reverse: GGTGAAGCGTTTGGAGT)

2.6. Statistical analyses

All data were analyzed by one-way ANOVA or two-way ANOVA using GraphPad Prism 5.0 (GraphPad Software Inc., USA). p Values < 0.05 indicated significant differences and were denoted by * or #. p Values < 0.01 indicated significant differences and were denoted by ** or ##. The results of real-time PCR were transformed to log10 and log2 values prior to the statistical analysis.

3. Results

3.1. Preparation of pGM-CSF and FliC protein

The sequence of the recombinant plasmid pSUMO-pGM-CSF and pSUMO-FliC was confirmed by restriction enzyme analysis and DNA sequencing (the data not shown). The pGM-CSF and FliC protein were expressed as soluble (Figs. 1A, 2 A), and the two protein were purified by affinity chromatograph of Ni^{2+}, respectively. SDS-PAGE analysis showed that the purified pGM-CSF protein was approximately 17 kDa (Fig. 1B) and the purified FliC protein was approximately 45 kDa (Fig. 2B).

3.2. Activity of pGM-CSF and FliC protein

MTT colorimetric method was used to compare the activity of GM-CSF standard and pGM-CSF protein in the TF-1 cells. Results demonstrated that standard sample and pGM-CSF protein had the similar activity to stimulate TF-1 cells proliferation and there was a good linear relationship between OD570 and protein concentration (Fig. 3A). Chickens were immunized, and serum was collected to detect NDV antibody titers by HI. The data demonstrated that there was almost no difference in maternal antibody titer of three groups in chickens, and the antibody level also did not show significant difference between three groups at 7 days after immunization. But at 14 and 21 days, NDV antibody titer of chickens in clone30 + 10 μg FliC group was higher than those in clone30 group and normal group (Fig. 3B). Consequently, pGM-CSF and FliC protein showed great biological activity.

3.3. Immunization study in mice

3.3.1. Humoral immune response in mice

Humoral immune response was assessed at 2, 4, 6, 8 and 10 days after immunization. The serum samples were collected to measure the PCV-specific antibody. As shown in Fig. 4, the level of anti-PCV antibody enhanced significantly from 2 to 6 days ($P < 0.05$ or $P < 0.01$) after immunization and began to reduce at 8 days ($P < 0.01$) in vaccine + pGM-CSF-treated mice, and the best dose was 25 ng pGM-CSF. The antibody level gradually increased in vaccine + FliC-treated mice, which were higher than those in vaccine group. Furthermore, mice in vaccine + pGM-CSF + FliC group showed higher antibody level than those in all other groups, which started to increase at 2 days ($P < 0.05$), reached the peak at 8 days ($P < 0.01$) and slowly decreased at 10 days ($P < 0.01$).

3.3.2. Cell-mediated immune response in mice

Hemocytes were collected at 2, 4, 6, 8 and 10 days after immunization and dissociated by ACK to get white blood cells, which were stained by CD3e-FITC antibody, CD4-PE antibody and CD8a-APC antibody to be measured by FCM. As shown in Fig. 5, the percentages of CD4+ and CD8 + T cells in mice of 25 ng pGM-CSF + vaccine group were higher than those of other groups in alone pGM-CSF group, which achieved the peak at 4 days ($P < 0.01$) after immunization, and then started to decrease. CD4+ and CD8 + T cells proliferation of vaccine + FliC-treated mice increased slowly at 2 days ($P < 0.01$) and reached the peak at 8 days ($P < 0.01$). The percentages of CD4+ and CD8 + T cells in mice of vaccine + pGM-CSF + FliC group were apparently higher than those in all other groups, which started to increase at 2 days ($P < 0.05$) and reached the highest level at 6 days ($P < 0.01$), in addition, CD4+ and CD8 + T cell numbers could continue to the tenth day after immunization.

3.4. Immunization study in piglets

3.4.1. Humoral immune response in piglets

Humoral immune response was evaluated at 2, 4, 6, 8, 10 and 13 days after immunization. PCV-specific antibody was detected by ELISA. As shown in Fig. 6, pigs in vaccine + pGM-CSF + FliC group showed the highest antibody level than those in all other groups ($P < 0.01$), and the antibody level was twice higher than pigs immunized with vaccine group at 2 days and 8 days ($P < 0.01$) which reached the peak at 8 days ($P < 0.01$). The antibody level of pigs in vaccine + pGM-CSF + FliC group still maintained the high condition at 13 days ($P < 0.01$), while the vaccine-treated pigs had no effect at 10 days and the antibody level was similar to those in PBS group. Pigs in vaccine + pGM-CSF group exhibited the highest antibody level at 6 days and the antibody level reduced at 8 days, which showed higher antibody level compared with the vaccine-treated pigs.

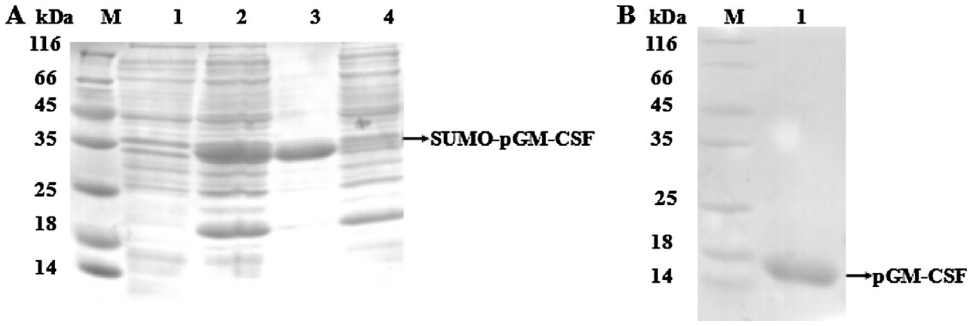

Fig. 1. Preparation of pGM-CSF. (A) SDS-PAGE analysis of the expression of pGM-CSF protein. Lane M: Unstained protein marker; Lane 1: Uninduced culture lysate; Lane 2: IPTG-induced culture lysate; Lane 3: IPTG-induced culture lysate, soluble fraction; Lane 4: IPTG-induced culture lysate, insoluble fraction. (B) SDS-PAGE analysis of purified pGM-CSF protein. Lane M: Unstained protein marker; Lane 1: Purified pGM-CSF protein.

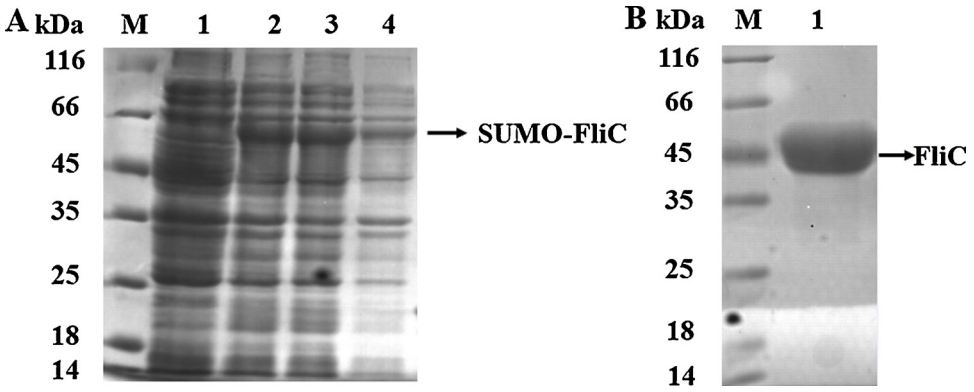

Fig. 2. Preparation of FliC. (A) SDS-PAGE analysis of the expression of FliC protein. Lane M: Unstained protein marker; Lane 1: Uninduced culture lysate; Lane 2: IPTG-induced culture lysate; Lane 3: IPTG-induced culture lysate, soluble fraction; Lane 4: IPTG-induced culture lysate, insoluble fraction. (B) SDS-PAGE analysis of purified FliC protein. Lane M: Unstained protein marker; Lane 1: Purified FliC protein.

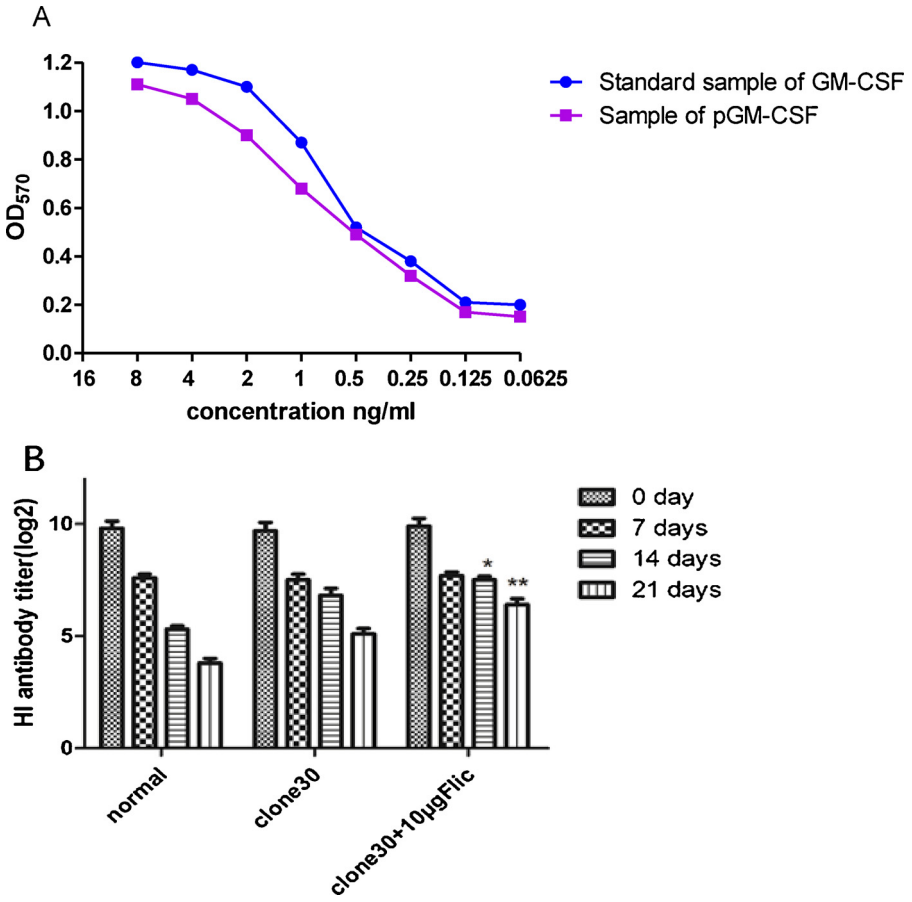

Fig. 3. Activity of pGM-CSF and FliC protein (A) Activity of pGM-CSF protein. (B) Activity of FliC protein. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs normal control.

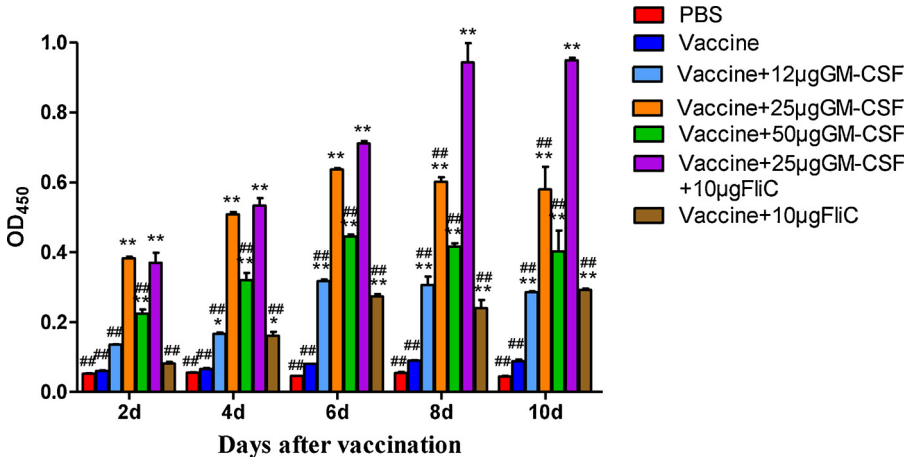

Fig. 4. Measurement of CPV-specific antibody by ELISA within 10 days after immunization in mice. The serum samples were collected at different days (2, 4, 6, 8 and 10 day). Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs vaccine-treated mice. ##P < 0.01 vs vaccine + pGM-CSF + FliC-treated mice.

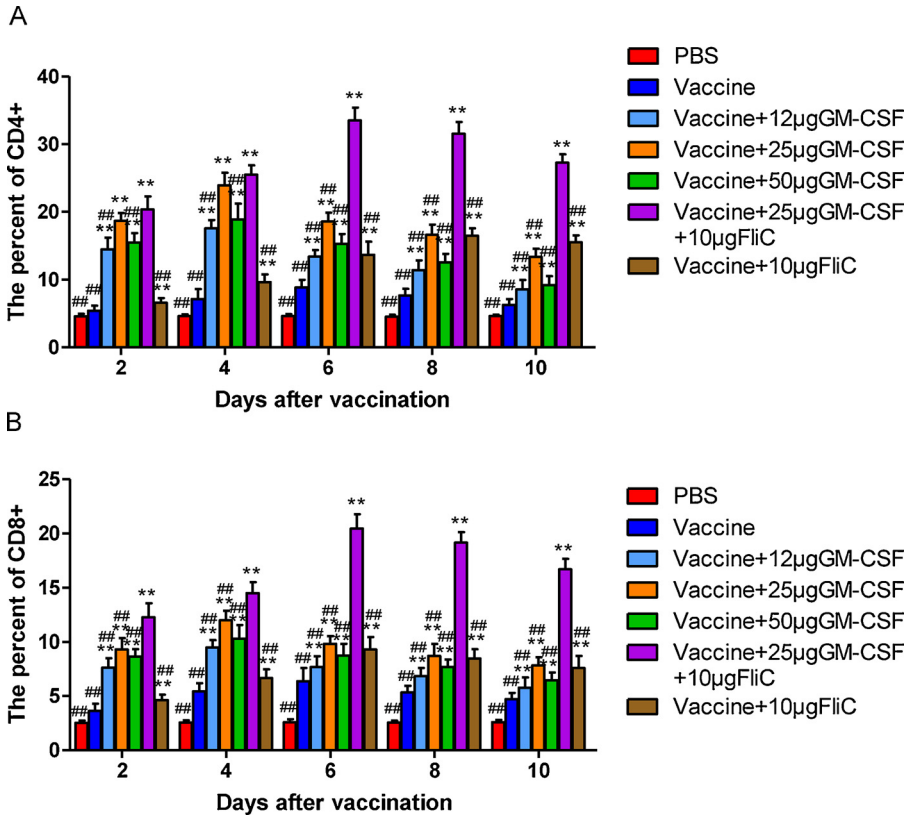

Fig. 5. Detection of CD4+ and CD8 + T numbers in mice. The peripheral blood was separated from immunized mice on 2, 4, 6, 8 and 10 days after vaccination. The percentages of CD4+ and CD8 + T numbers were detected by FCM. (A) FCM analysis of the CD4 + T-cell; (B) FCM analysis of CD8 + T-cell. Data are expressed as mean ± SD. **P < 0.01 vs vaccine-treated mice. ##P < 0.01 vs vaccine + pGM-CSF + FliC-treated mice.

3.4.2. Cell-mediated immune response in piglets

White blood cells were isolated from the hemocytes of pigs which were collected at 2, 4, 6, 8 and 10 days after immunization. The percentages of CD3+ and CD4+/CD8+ double positive T cells were detected by FCM. As shown in Fig. 7, the percentages of CD4 + T cells in pigs of vaccine + pGM-CSF + FliC group were twice higher than pigs immunized with vaccine group at 2 days (P < 0.01) and achieved to the peak at 6 days (P < 0.01). The percentages of CD8 + T cells in pigs of vaccine + pGM-CSF + FliC group were three times higher than pigs immunized with vaccine group at 2 days (P < 0.01) and reached to the peak at 8 days (P < 0.01). Pigs in vaccine + pGM-CSF + FliC group still exhibited high CD4+ and CD8 + T cells levels, while the vaccine-treated pigs had scarcely effect at 10 days after immunization, in particularly, CD4 + T cells of pigs in vaccine + pGM-CSF + FliC group were four times higher and CD8 + T cells of pigs in vaccine + pGM-CSF + FliC group were five times higher compared with the pigs in vaccine group (P < 0.01). The percentages of CD4+ and CD8 + T cells in pigs of vaccine + pGM-CSF + FliC group still were significantly higher than those in all other groups (P < 0.01), which in pigs of vaccine + pGM-CSF group were dramatically higher than those in vaccine group from 2 to 10 days (P < 0.01).

Cytokine IL-1, IL-8 and IL-17 were analyzed by real-time PCR. IL-1, IL-8 and IL-17 had the similar rules in the transcriptional level of mRNA (Fig. 8), which had a rapid up-regulation at 2 days and instant down-regulation at 4 days in vaccine + pGM-CSF and vaccine-treated pigs, while rising slightly 4 days after immunization in vaccine + pGM-CSF + FliC-treated pigs. mRNA level of IL-1, IL-8 and IL-17 in pigs of vaccine + pGM-CSF + FliC group and vaccine + pGM-CSF group was higher than those in vaccine group at 8 and 10 days (P < 0.01).

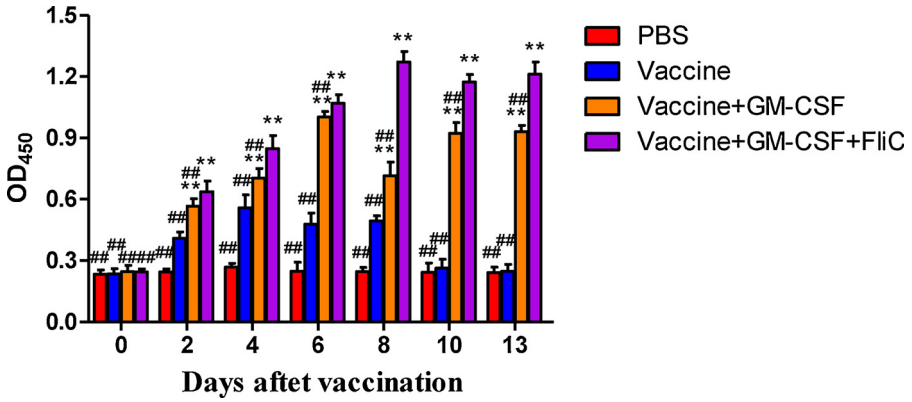

Fig. 6. Measurement of CPV-specific antibody by ELISA within 10 days after immunization in pigs. The serum samples were collected at different days (0, 2, 4, 6, 8, 10 and 13 day). Data are expressed as mean ± SD. **P < 0.01 vs vaccine-treated pigs. ##P < 0.01 vs vaccine + pGM-CSF + FliC-treated pigs.

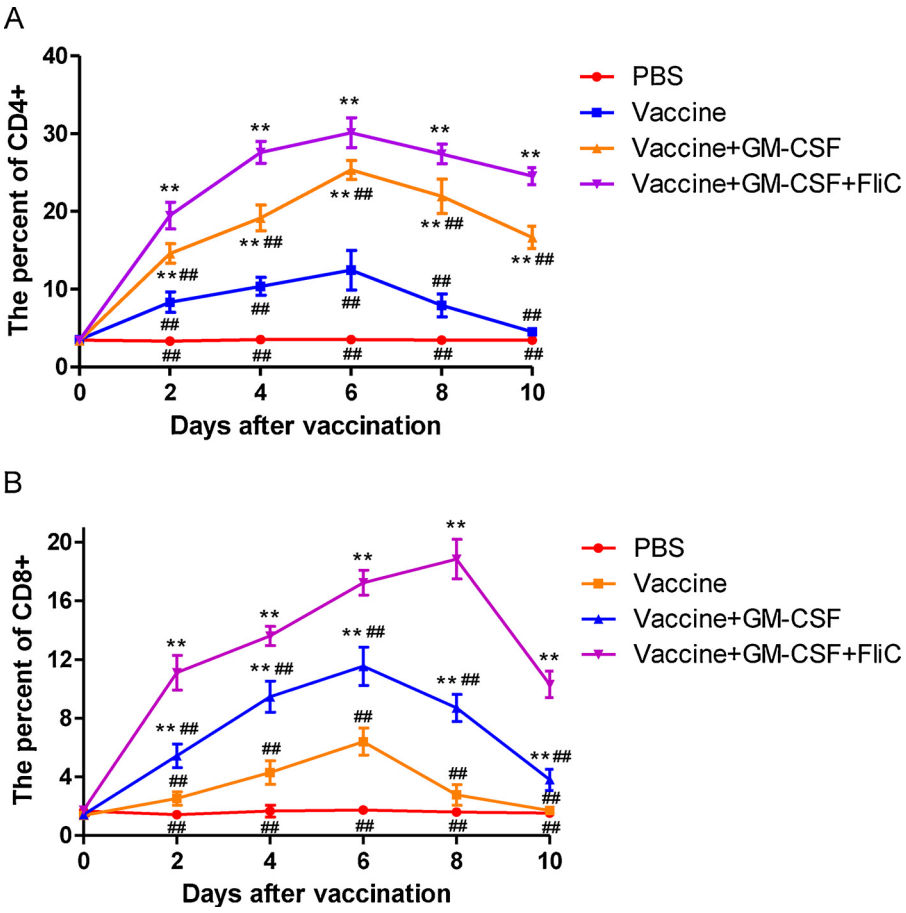

Fig. 7. Detection of CD4+and CD8 + T numbers in pigs. The peripheral blood was separated from immunized pigs on 2, 4, 6, 8 and 10 days after vaccination. The percentages of CD4+ and CD8 + T numbers were detected by FCM. (A) FCM analysis of the CD4 + T-cell; (B) FCM analysis of CD8 + T-cell. Data are expressed as mean ± SD. **P < 0.01 vs vaccine-treated pigs. ##P < 0.01 vs vaccine + pGM-CSF + FliC-treated pigs.

4. Discussion

Vaccination is the most widely used control strategy for PCV in many countries (Beach and Meng, 2012). Although with the constantly emerging of the new type vaccines, for example, DNA vaccine or subunit vaccine, which could not effectively combat viral infections (Renukaradhya et al., 2015; Schijns and Lavelle, 2014). Many vaccines require to enhance its immunogenicity with the help of the immune adjuvants that can improve the effect of immune responses (Reed et al., 2013; Petrovsky and Aguilar, 2004; Mbow et al., 2010; Sivakumar et al., 2011). Therefore, adjuvant plays an important role in vaccines development, it can induce humoral and cell-mediated immune responses and its immune enhancing function has been well proven in many studies (Brown et al., 2014; Wack et al., 2008). However, there are fewer studies on the composite biological adjuvants, and the joint application of different kinds of vaccine adjuvants might be an effective way to increase the immune effect markedly. GM-CSF and FliC can directly stimulate the activation and maturation of DCs and enhance the expression of T cells co-stimulatory signals and Ag-presenting molecules (Means et al., 2003; Shi et al., 2006). Meanwhile, GM-CSF and FliC induce monocytes and NK cells to produce cytokines and stimulate T cells to proliferate and produce cytokines and chemokines, which enhance the innate and adaptive immune (Burgess and Metcalf, 1980; Caron et al., 2005; Farina et al., 2004). Therefore, GM-CSF and FliC protein were selected as composite biological adjuvants to enhance immune response against PCV.

We measured the activity of the purified pGM-CSF in vitro, comparing with the GM-CSF standard, pGM-CSF showed identical activity

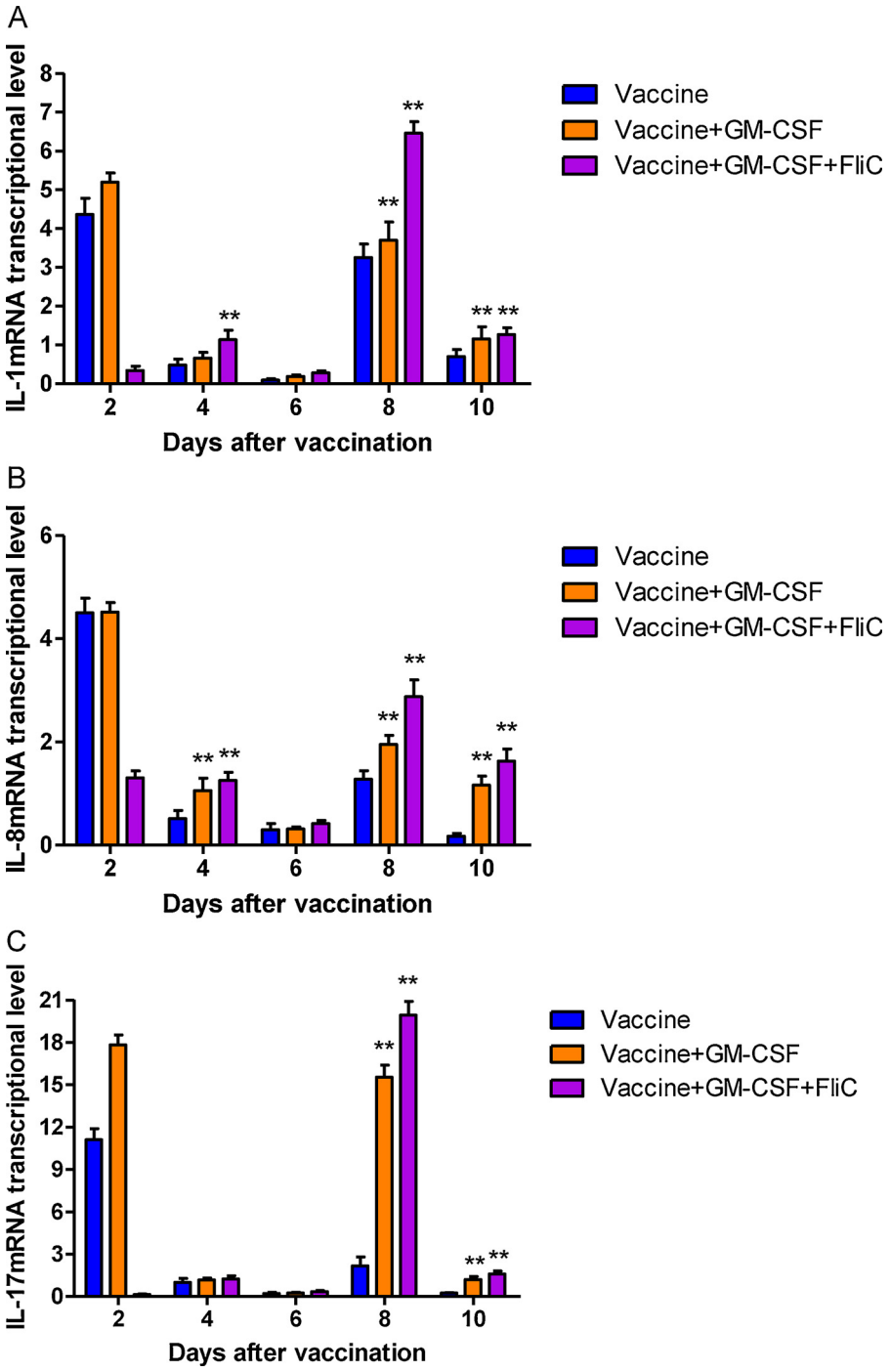

Fig. 8. The mRNA transcriptional level of cytokine in pigs. (A) The mRNA transcriptional level of IL-1. (B) The mRNA transcriptional level of IL-8. (C) The mRNA transcriptional level of IL-17. Data are expressed as mean ± SD. **P < 0.01 vs vaccine-treated pigs.

level on maintaining the growth of TF-1 cells. Simultaneously, we detected the activity of the purified FliC protein in vivo, then, we immunized the chickens with FliC protein and clone30 vaccine to improve the immune effect of NDV vaccine, the results showed that FliC protein could be used as an immune adjuvant to enhance the immune effect of clone30 vaccine and it has fine biological activity. Because the pGM-CSF sequence exhibits about 70% homologous identity to the mouse GM-CSF sequence and mouse is small in volume which is easier to operate, so the immune effect of adjuvants (pGM-CSF and FliC protein) were measured with PCV vaccine in mice firstly. The results showed that mice in vaccine + pGM-CSF + FliC group had significantly higher PCV specific antibody level and CD4+ and CD8 + T-cell numbers than those in vaccine + pGM-CSF and vaccine + FliC group, particularly at 8 and 10 days after immunization, indicating that when GM-CSF and FliC used together, they both enhanced the immune response level and might have potential synergies effect. Moreover, the presence of the pGM-CSF and FliC adjuvants effectively stimulated T cells and B cells responses and had a good biological activity on mice in vivo.

According to the results of immunization with mice, the immune effect of pGM-CSF and FliC adjuvants was detected with PCV vaccine in pigs. The optimum dose of pGM-CSF was selected which was $1.25\,\mu g\,kg^{-1}$. The level of PCV specific antibody of pigs immunized with vaccine + pGM-CSF group rose rapidly and slightly decreased and keep stable subsequently, which were higher than those in vaccine

group. Whereas, the antibody level of pigs in vaccine + pGM-CSF + FliC group was significantly higher than those in all other groups after immunization, which could keep higher antibody level at 13 days, but pigs in vaccine group could not work at 10 days. Those results demonstrated that both GM-CSF and Flic adjuvants stimulated humoral immune response to produce the specific antibody more efficiently compared with vaccine group, which could greatly improve the immune effect of vaccine. The levels of CD4+ and CD8 + T-cell response of pigs immunized with vaccine + pGM-CSF + FliC group were higher than those in all other groups apparently after immunization, suggesting that the pGM-CSF and FliC adjuvants stimulated cellular immune response quickly and significantly compared with vaccine group. CD4 + T-cell is very important factor in the cellar and humoral immune and it can activate B cell to produce antigen-specific antibody (Lund and Randall, 2010). In addition, induction of CD8 + T-cell responses requires the presence of CD4 + T-cell to stimulate dendritic cells and provide cytokines (Lanzavecchia, 1998; Schuurhuis et al., 2000). Hence, GM-CSF and FliC had significance for enhancing the activation and proliferation of CD4 + T cells. CD4+ and CD8 + T cells numbers increased was consistent with the observations of IL-1, IL-8 and IL-17 transcriptional levels. Interleukin (IL-1, IL-8 and IL-17) can promote thymocytes and T cells to activate, proliferate and differentiate and enhance NK cell kill activity (Garlanda et al., 2013; Moseley et al., 2003; O'Shea and Murray, 2008), however, the transcriptional level of IL-1, IL-8 and IL-17 was up-regulated obviously at 8 and 10 days after immunization, which might contribute to the rise in T cells numbers. Therefore, our results clearly showed that use of pGM-CSF and FliC as compound adjuvants with PCV vaccine markedly increased humoral immune response and cell-mediated immune response in pigs.

In summary, those results demonstrated that pGM-CSF and FliC as composite biological adjuvants of the vaccine showed a stronger immune response compared with alone adjuvant and alone vaccine groups which were powerful immunoadjuvant to potentiate quickly humoral and cellular immune responses in mice and pigs. However, the two adjuvants GM-CSF and FliC can be used as an effective and safe porcine vaccines candidate for preventing PCV, which provide insight into the development of new type of safety and high efficiency porcine vaccine adjuvant, and we can apply the compound adjuvants in other animals.

Conflict of interest statement

The authors declare that they have no competing interests.

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