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Virus Research 247 (2018) 84–93

Contents lists available at ScienceDirect

Virus Research

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

Construction and immunogenicity analysis of *Lactobacillus plantarum* expressing a porcine epidemic diarrhea virus S gene fused to a DC-targeting peptide

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ARTICLE INFO

Keywords:
DCpep
PEDV
S protein
L. plantarum
Immune response

ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is one of the most important causative pathogens of swine diarrhea, which is widely prevalent throughout the world and is responsible for significant economic losses in the commercial pig industry, both domestic and abroad. The spike (S) protein in the PEDV capsid structure can carry the major B lymphocyte epitope, which induces production of neutralizing antibodies and provides immunoprotective effects. Moreover, the conserved region encoded by the S gene can be considered a target for establishing a new diagnostic method and is a new candidate for vaccine design. In this study, use of anchorin pgsA' allowed the fusion protein of S-DCpep to express on the surface of recombinant *Lactobacillus plantarum* (NC8-pSIP409-pgsA'-S-DCpep) NC8 strain. Mice were immunized by lavage administration of the recombinant NC8-pSIP409-pgsA'-S-DCpep, which was observed to induce DC activation and high production of sIgA and IgG antibodies in experimental animals, while also eliciting production of significantly more IgA^+B220^+ B cells. More importantly, secretion of cytokines IFN-γ, IL-4 and IL-17 in mice that were vaccinated with NC8-pSIP409-pgsA'-S-DCpep was remarkably increased. The results of our study suggest that NC8-pSIP409-pgsA'-S-DCpep potently triggers cellular and humoral immune responses. The obtained experimental results can provide a theoretical basis that lays the foundation for production of a novel oral vaccine against PED.

1. Introduction

Porcine epidemic diarrhea (PED) is a highly contagious disease with typical symptoms in piglets that include severe diarrhea, vomiting and intestinal dehydration (Ko et al., 2017). The morbidity and mortality rates of suckling piglets caused by PEDV are more than 50%, and it is the primary cause of death in piglets and weight loss in fattening pigs (Choi et al., 2009). This viral disease was first reported in Britain in 1971 and then became an epidemic in other European countries (Yu et al., 2017). In 1976, PEDV was detected for the first time in China, and since October 2010, PEDV outbreaks worldwide have become more frequent (Song et al., 2015). For instance, PED broke out in Minnesota and Iowa in the United States in 2013, resulting in huge losses to the pig industry (Diep et al., 2017; Lee, 2015). As a coronavirus, PEDV has a typical structure that consists of four structural proteins, including the nucleocapsid protein (N), a membrane protein (M), a glycosylated spike protein (S) and a envelope protein (E) (Kocherhans et al., 2001). The PEDV spike protein-encoding gene is an immune dominant gene that encodes a protein with a very dense hydrophobic region of amino acids that harbors multiple glycosylation sites and has a high antigenic index (Li et al., 2017a). Several studies have reported that the S protein is a structural surface protein of PEDV, containing both the receptor binding domain, which mediates invasion of the virus into the host cell, and the antigenic epitope, which mediates production of the neutralizing virus antibody (Zhang et al., 2016). Thus, the S-glycoprotein plays a crucial role in viral infection, pathogenicity and adsorption. It has been shown that the S-glycoprotein is immunogenic and has the potential to be a good vaccine candidate (Fan et al., 2015).

Lactic acid bacteria (LAB) are diverse species that are widely distributed in nature and produce large amounts of lactic acid by

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https://doi.org/10.1016/j.virusres.2017.12.011
Received 30 September 2017; Received in revised form 21 December 2017; Accepted 21 December 2017
Available online 27 December 2017
0168-1702/

fermenting carbohydrates (Riaz Rajoka et al., 2017). LAB, which are generally recognized as safe bacteria, colonize the intestines of humans or animals and have potentially beneficial effects (Landete et al., 2015). The study of LAB has many advantages, including that they are easy to culture, convenient to manipulate and highly safe, which is why LAB can be regarded as a heterologous antigen protein delivery system (Wanker et al., 1995). *L. plantarum* has been manipulated to deliver a number of viral antigens in previous studies (Li et al., 2017b; Yang et al., 2017c). The *L. plantarum* strain NC8 was isolated from ensilages and has a better antireversion characteristic compared with LAB isolated from healthy animal intestines (Caggianiello et al., 2016; Park et al., 2017). Thus, *L. plantarum* NC8 is more suitable to act as a mucosal delivery shuttle vector to express protective antigens.

Recently, poly-γ-glutamic acid synthetase A (pgsA) was identified as being a constitutively expressed protein of the polyglutamate acid (PGA) synthetase system of *Bacillus subtilis*. PgsA can stably anchor γ-PGA synthetase on the cell membrane, acting as a transport carrier of γ-PGA and playing a crucial role in the formation of γ-PGA (Sung et al., 2005). The pgsA protein has a transmembrane region near its N-terminus (26–42 amino acid residues), providing the necessary characteristics for the establishment of a pgsA transmembrane expression system. The pgsA gene was combined with the target gene for fusion expression, after which *L. plantarum* was used as a mucosal vehicle to deliver an exogenous antigen. This experiment provided a theoretical basis for the construction of a novel, orally administered, genetic engineered vaccine to immobilize exogenous medicinal proteins on the cell wall surface of *L. plantarum* (Cai et al., 2016; Narita et al., 2006).

Dendritic cells (DCs) are the most powerful functional antigen presenting cells (APCs) and are the exclusive APCs that are capable of inducing initial T and B cell activation (Yang et al., 2016). DCs can elicit T cell polarization and differentiation in T cells subsets, which also trigger secretion of IFN-γ (Subramaniam et al., 2017). DCs are involved in regulating the function of B cells and promoting the differentiation of B cells into plasma cells to produce IgG (Wang et al., 2016). In the germinal center, DCs can boost B cell antibody affinity maturation and help to form and maintain memory B cells. In addition, sIgA can play an important role in maintaining intestinal integrity and intestinal symbioses (Shaw et al., 2000). DCs can restrict penetration of microorganisms into the mucosa and promote uptake of antigens in the intestinal mucosal system (Owen et al., 2013). It has been reported that NC8-pSIP409-pgsA'-S-DCpep can specifically conjugate dendritic cell-targeting peptide (DCpep) and induce an immune response (Apostolico et al., 2017). To make DCs recognize the S-antigen more effectively in the intestinal mucosa, recombinant NC8-pSIP409-pgsA'-S-DCpep was constructed using standard molecular biology techniques, and its immunogenicity was further investigated. After BALB/c mice were immunized by lavage administration of our recombinant *L. plantarum*, the results indicated that the in vivo antibody levels were greatly improved.

2. Materials and methods

2.1. Construction of NC8-pSIP409-pgsA'-S-DCpep using gene recombination

The *Escherichia coli-Lactobacillus* shuttle vector pSIP409 (Sorvig et al., 2005) and *L. plantarum* NC8 were kindly provided by Professor A. Kolandaswamy (Madurai Kamaraj University, India). The pM18-T-S-DCpep (DC peptide, FYPSYHSTPQRP) was stored at Jilin Provincial Engineering Research Center of Animal Probiotics in Jilin Agricultural University. The pM18-T-S-DCpep plasmid was cut at *Xba*I and *Hind*III sites within the primers using the appropriate restriction enzymes at 37 °C for 3–4 h. Next, the digested 2240 bp S gene fragment (Spike Protein Gene GenBank: JQ257007.1 (source: 75–2313)/Spike Protein GenBank: AEW24858.1 (source: 26–771)) and DCpep (S-DCpep) were ligated into pSIP409-pgsA' using T4 DNA ligase at 16 °C overnight. The recombinant pSIP409-pgsA'-S-DCpep plasmid was detected and purified using a gel extraction kit (Axygen). The recombinant plasmid was introduced into the *L. plantarum* NC8 strain by electroporation and erythromycin-resistant bacteria were selected for, after which the plasmid was sequenced. In addition, to meet the rigorous nature of the experimental study, a NC8-pSIP409-pgsA'-S-Ctrlpep (control peptide, EPIHPETTFTNN) was constructed similar to NC8-pSIP409-pgsA'-S-DCpep and used as the experimental control group.

2.2. Immunofluorescent identification

To examine the expression of the S-protein on the surface of recombinant NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep was cultured in MRS with 10 μg/ml erythromycin. As described in previous studies, we added Sakacin P (SppIP) to the culture medium when the (OD_{600}) of the medium was between 0.3 and 0.4. After induction at 37 °C for 10 h, cells from each strain were washed with PBS containing 1% bovine serum albumin. Next, cells were resuspended in PBS and 1×10^5 CFU of bacteria were incubated with 1 ml of primary antibody (1/2000 anti-S-produced in rat) for 1 h at 37 °C with shaking at 60 rpm. The pellets were washed with PBS containing 0.2% Tween-20 (PBS-T), after which secondary antibodies (FITC, goat anti-rat IgG (H + L), CST) were added and the mixture was incubated for one hour at 37 °C with shaking at 60 rpm in the dark. Finally, samples were washed with PBS-T and resuspended in PBS. The cells were examined under a laser scanning confocal microscope (LSM710; Carl Zeiss, Germany) following the manufacturer's instructions.

2.3. Western blot analysis

To detect the S-protein expression levels, we induced its expression in recombinant LAB as described previously. Strains were subsequently resuspended in 500 μl of PBS and were lysed by ultrasonic crushing as previously described (Shi et al., 2014). Samples were assessed by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The S-Ctrlpep and S-DCpep proteins were detected using an anti-S polyclonal primary antibody (polyclonal antibody was obtained from serum of mice which were immunized with the purified S protein) and a HRP-conjugated goat anti-rat IgG secondary antibody (A21040; Abbkine, USA). The bands were visualized using enhanced chemiluminescence reagents in an ECL Plus detection kit (Thermo Scientific) according to the manufacturer's instructions.

2.4. Ethics statements of experimental animals

For experiments involving animals, thirty-six female BALB/c mice without specific pathogen (SPF) were bought from Beijing HFK Bioscience Co., Ltd., China. According to the provisions of the Animal Care and Ethics Committees of Jilin Agriculture University. All animals were strictly raised with commercialized germ-free feed and sterilized water in the Jilin Provincial Engineering Research Center of Animal Probiotics, which provided the pathogen-free environment facilities.

2.5. Immunization

Six-week-old mice with no specific pathogens were randomly divided into four groups, with 6 mice in each group (n = 9). Briefly, using oral gavage, four groups of mice were immunized with NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep, NC8-pSIP409-pgsA'-S-DCpep or saline. The entire immunization procedure was divided into three phases: prime immunization (days 1–3), booster immunization (days 8–10), and the last immunization (days 29–31), with 10^8 CFU/mouse administered by oral gavage.

2.6. Preparation of single cell suspension

Generally, experimental mice were sacrificed by cervical dislocation

30 days after being immunized. Lamina propria (LP) cells of the small intestine, PPs and MLNs were acquired via a previously described method (Kikuchi et al., 2014) on a clean sterile clean bench. Small intestine samples were washed with PBS and cut into small pieces. Next, samples were digested with lymphocyte separation medium, after which LP cell digestive juice was added to obtain single LP cells from the small intestine in an isotonic percoll solution. The cell suspension was filtered through a 70 μm sterile filter-film and the filtrate was centrifuged at 500*g* for 5 min at 4 °C. Subsequently, the cells were washed with 4 °C PBS and were eventually resuspended in complete RPMI 1640 (containing 10% FBS, 1% penicillin and streptomycin).

2.7. Flow cytometry

The method used for the analysis of S protein expression in recombinant *L. plantarum* was consistent with the immunofluorescence assay, with cells analyzed by flow cytometry (BD FACS LSR Fortessa™ Flow Cytometer). Data were analyzed via FlowJo 7.6.1 software. To test the activation of DC surface costimulatory molecules and B cells, flow cytometry was conducted using a previously published protocol (Shi et al., 2016). In brief, single LP cells from the small intestine suspensions obtained from immunized mice were diluted to 1×10^6 cells/100 μl and incubated with 10 μl anti-CD11c (clone HL3), CD80 (clone 16–10A1), CD86 (clone GL1), CD40 (clone 3/23) and MHCII (clone 3/23) antibodies to assess the DC cells. To analyze B lymphocyte cells activation, 10 μl of anti-B220 (clone RA3-6B2) and IgA (clone C10-3) antibodies was added to stain B lymphocyte cells for 20 min at room temperature in the dark. Afterwards, cell suspensions were washed twice with FACS buffer. After staining, samples were examined using flow cytometry (BD FACS LSR Fortessa™ Flow Cytometer). Data were analyzed using FlowJo 7.6.1 software.

2.8. ELISA

Blood samples were obtained on days 14, 35 and 42 after immunization and were centrifuged at 4000 rpm for 15 min at 4 °C to obtain the serum, which was stored at −80 °C. The fecal samples were prepared according to our previously published method, followed by centrifugation at 5000 rpm for 20 min at 4 °C. The supernatants were acquired and stored at −80 °C for further analysis. ELISA was performed to determine the levels of specific IgG and sIgA antibodies (Yang et al., 2017d). In brief, 100 μl of purified S protein (4 μg/ml) was added to each well of the 96-well plate and incubated overnight at 4 °C. On the second day, the blocking solution was added and the plate was incubated overnight at 4 °C. On the third day, the fecal samples (1:10) and serum samples (1:100) were diluted and added separately, after incubating at 37 °C for 2 h, the wells were rinsed. Subsequently, goat anti-mouese IgG (H + L)-Biotin and goat anti-mouse IgA-Biotin (Southern Biotechnology, Birmingham, AL) was added separately, and the plate was incubated at 37 °C for one hour, after which Streptavidin-HRP (Southern Biotechnology, Birmingham, AL) secondary antibody was added and the plate was incubated at 37 °C for 30 min. Finally, TMB substrate solution and stop solution was added, after which the color intensity was measured at 492 nm. The End-point titers (log2) were defined as the highest dilution yielding an absorbance that was two and three times higher than the background for the fecal and serum samples, respectively (Shi et al., 2016).

2.9. Detection of cytokines

The MLN cells were stimulated by S protein (5 μg/ml) and the supernatant was collected 3 days later and examined for specific cytokines, including IFN-γ, IL-4 and IL-17A using an ELISA kit (LIUHEBIO, Wuhan, China) according to the manufacturer's instructions.

2.10. Cell neutralization test (fixed virus dilution serum method)

For determining the PEDV neutralizing activity of the serum antibodies we detected sample obtained from mice which were orally immunized with recombinant *L. plantarum* by antibody neutralization assay (microdetermination) as described before (Tian et al., 2014) with minor modifications. Under aseptic conditions serum and fecal samples were collected at 14, 35 and 42 days respectively after immunization, quantitative virus and serial diluted serum were added to Vero cells to evaluate serum neutralizing antibody titers, the neutralizing antibody titer PD50 (Median protective dose refers to the dose of serum or vaccine that protects half (50%) of the experimental animals) was determined by Karber's method at 72 h.

2.11. Statistical analysis

All results were performed by Two-tailed *t*-tests and One-way analyses of variance (ANOVA) (GraphPad Prism 5.0, GraphPad Software) were used to analyze the significance of the difference between means. All data were obtained from at least three independent experiments, and the geometric mean titers were expressed as averages. All data were expressed as the means ± SEM.

3. Results

3.1. Expression of PEDV S protein on L. plantarum

The pSIP409-pgsA'-S-DCpep and pSIP409-pgsA' plasmids were introduced separately into *L. plantarum* NC8 strain by electroporation (Fig. 1A). Using flow cytometry, S protein expression levels of recombinant *L. plantarum* NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep were determined (Fig. 1B). The green fluorescence marked by white arrows indicated that recombinant *Lactobacillus* of NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep expressed S protein on the surface (Fig. 1C) than NC8-pSIP409-pgsA', which was barely observed with green fluorescence under a confocal microscope. Western blot analysis showed that a specific band appeared at 80 kDa, which demonstrated that the PEDV S protein had been expressed. Thus, the experimental results suggest that NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep were expressed in *L. plantarum* NC8.

3.2. Activation of DC surface molecules elicited by NC8-pSIP409-pgsA'-S-DCpep

To determine the effect of NC8-pSIP409-pgsA'-S-DCpep on DCs, flow cytometry was performed to test the level of costimulatory molecules activated by DCs, which were induced by NC8-pSIP409-pgsA'-S-DCpep gavage immunization 3 days after the last immunization (Fig. 2A). Compared with the saline group, the expression level of $CD11c^+CD40^+$ of DCs surface molecules in the LP cells of the small intestine was significantly increased in the NC8-pSIP409-pgsA'-S-DCpep group ($P < 0.01$) and NC8-pSIP409-pgsA'-S-Ctrlpep group ($P < 0.05$) experimental groups (Fig. 2B). Moreover, our experimental results indicated that NC8-pSIP409-pgsA'-S-DCpep can promote greater expression of $CD80^+$ in LP DCs than the NC8-pSIP409-pgsA'-S-Ctrlpep ($P < 0.05$), NC8-pSIP409-pgsA' ($P < 0.05$) or saline ($P < 0.01$) groups (Fig. 2C). However, no obvious differences were observed in $CD11c^+CD86^+$ expression of DCs surface molecules in the LP cells of the small intestine.

3.3. Influence of NC8-pSIP409-pgsA'-S-DCpep on the B cell immune response

The number of IgA^+B220^+ B cells in PP was determined by flow cytometry 3 days after the last immunization (Fig. 3A). The results

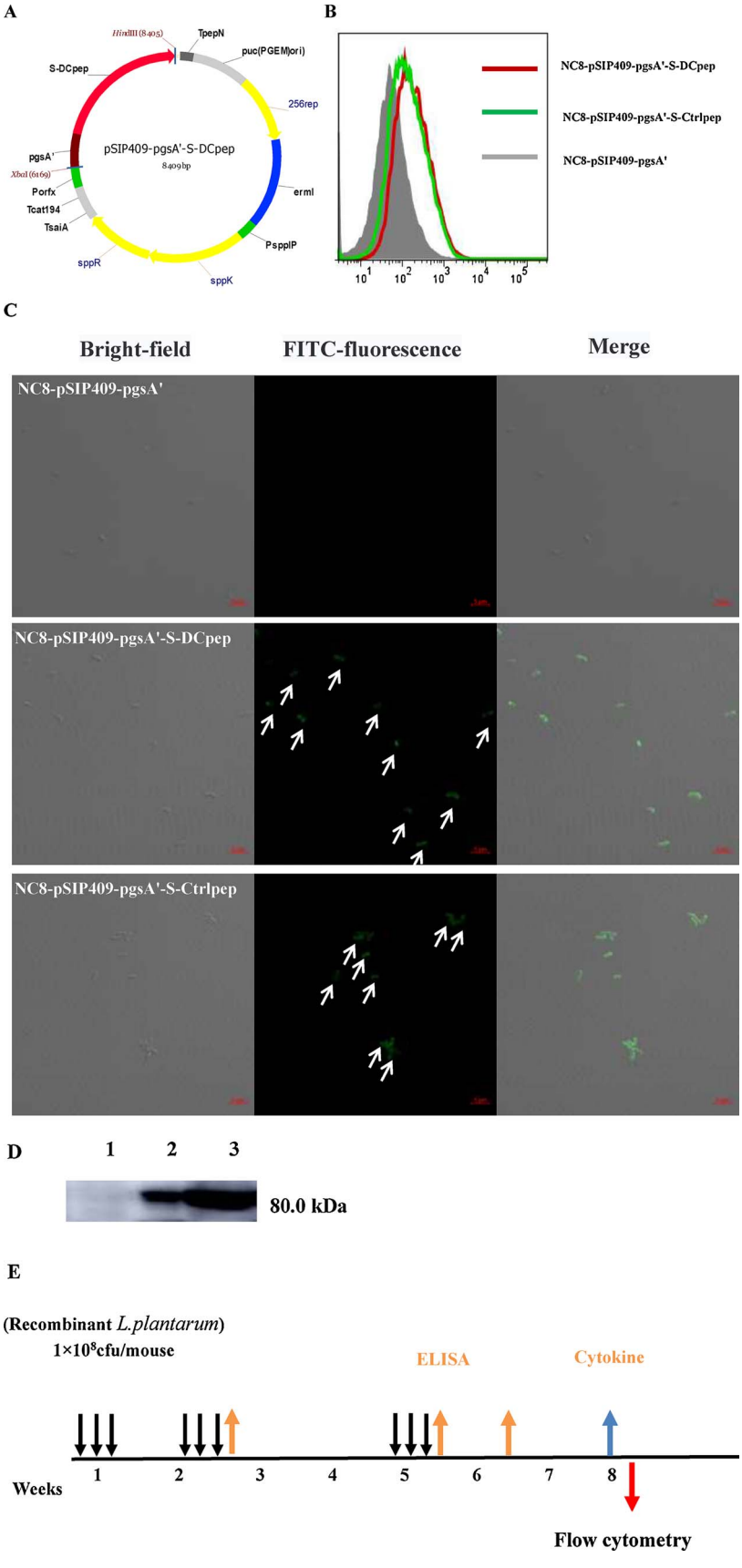

Fig. 1. Construction and expression of NC8-pSIP409-pgsA'-S-Dcpep. (A) Schematic diagram of the NC8-pSIP409-pgsA'-S-DCpep plasmid. (B) Flow cytometry (the red shows NC8-pSIP409-pgsA'-S-DCpep, the green shows NC8-pSIP409-pgsA'-S-Ctrlpep, the gray shows NC8-pSIP409-pgsA') and (C) immunofluorescence confocal microscopy detection of the PEDV S protein expression (the wihte arrows indicate the green fluorescence for the expressed S protein). (D) Western blot analysis of recombinant NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep expression using an anti-S polyclonal antibody. Lane 1 shows NC8-pSIP409-pgsA', Lane 2 shows NC8-pSIP409-pgsA'-S-Ctrlpep, Lane 3 shows NC8-pSIP409-pgsA'-S-DCpep. (E) Protocol for the mouse experiment. The mice were lavage administered NC8-pSIP409-pgsA'-S-DCpep, NC8-pSIP409-pgsA'-S-Ctrlpep, NC8-pSIP409-pgsA' (1×10^8cfu in 200 µl per mouse), or saline (200 µl per mouse) on days 1–3, 8–10, 29–31. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

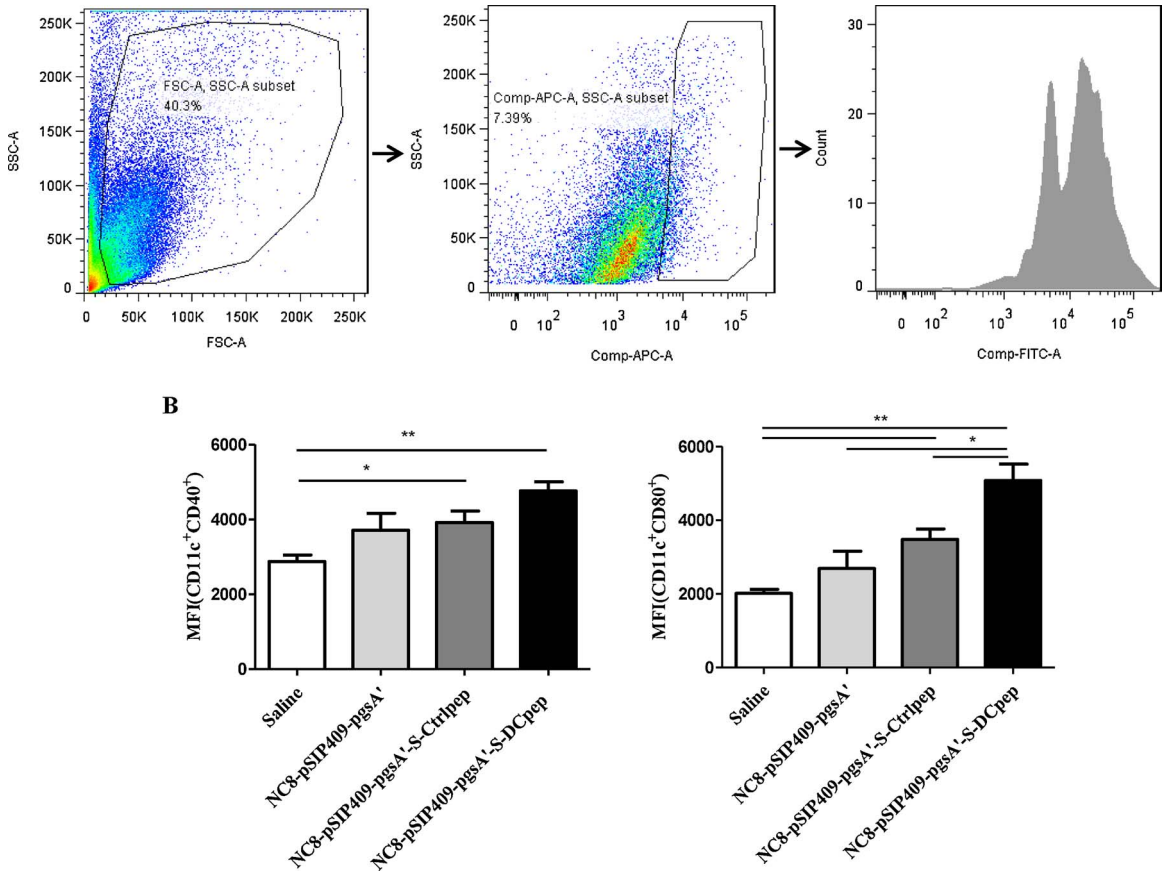

Fig. 2. Analysis of DCs surface co-stimulatory molecules in the LP of the small intestine increased expression of $CD80^+$ and $CD40^+$. Effects of NC8-pSIP409-pgsA'-S-DCpep on DCs activation were detected by flow cytometry. (A) Gate process of $CD11c^+CD80^+$ and $CD11c^+CD40^+$ double positive expression in LP of small intestine cells. (B) The mean fluorescence intensity (MFI) values in the bar graphs refers to the activated DCs levels. The values are presented as the percentages of activated DCs expressing the markers $CD11c^+CD40^+$ and $CD11c^+CD80^+$. Significant differences are denoted by an asterisk (*) between Stroke-physiological saline solution, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

showed that there was a remarkable increase in the percentage of IgA^+B220^+ B cells within the B cell populations of BALB/c mice having received lavage administration with NC8-pSIP409-pgsA'-S-DCpep compared with the NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and saline groups. In addition, NC8-pSIP409-pgsA'-S-DCpep in PP significantly increased the production of IgA^+B220^+ B cells compared to the NC8-pSIP409-pgsA' and saline group control ($P < 0.01$) (Fig. 3B), and the NC8-pSIP409-pgsA'-S-DCpep was significantly higher than the NC8-pSIP409-pgsA'-S-Ctrlpep groups in PP ($P < 0.05$) (Fig. 3B). Collectively these results demonstrated that vaccination with the NC8-pSIP409-pgsA'-S-DCpep can activate the B cell response in mice.

3.4. NC8-pSIP409-pgsA'-S-DCpep elicited mucosal sIgA secretion

The specific sIgA levels in fecal intestines were measured at days 14, 35 and 42 after the first immunization using ELISA (Fig. 4). However, the concentration of sIgA in the excrement of mice treated with NC8-pSIP409-pgsA'-S-DCpep was higher than that of the NC8-pSIP409-pgsA' ($P < 0.05$) and saline ($P < 0.05$) groups at days 14 (Fig. 4), and NC8-pSIP409-pgsA'-S-DCpep was higher than that of the NC8-pSIP409-pgsA' ($P < 0.05$) and saline ($P < 0.01$) groups at day 35 (Fig. 4). In contrast, no obvious difference was observed with respect to SlgA was observed at days 14 and 35 in the NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep groups (Fig. 4). With respect to the 42 day lavage administration, the NC8-pSIP409-pgsA'-S-DCpep group was higher than that of the NC8-pSIP409-pgsA' ($P < 0.01$) and saline ($P < 0.01$) groups. Higher expression of sIgA was also observed in NC8-pSIP409-pgsA'-S-DCpep group compared with NC8-pSIP409-pgsA'-S-Ctrlpep group ($P < 0.05$) at day 42 (Fig. 4). These data suggested that lavage administration of NC8-pSIP409-pgsA'-S-DCpep could stimulate the mucosal immune system in mice.

3.5. NC8-pSIP409-pgsA'-S-DCpep triggered serum IgG production

The specific IgG-antibodies level after lavage administration of NC8-pSIP409-pgsA'-S-DCpep in mice was notably increased compared with the NC8-pSIP409-pgsA ', NC8-pSIP409-pgsA'-S-Ctrlpep and saline groups at days 14, 35 and 42 after the first immunization. The results showed that the concentration of specific IgG in the serum of mice treated with NC8-pSIP409-pgsA'-S-DCpep was significantly increased compared to the NC8-pSIP409-pgsA' ($P < 0.05$) and saline ($P < 0.01$) groups at days 14 and 35 (Fig. 5). In addition, only NC8-pSIP409-pgsA'-S-DCpep showed higher levels than the NC8-pSIP409-pgsA'-S-Ctrlpep ($P < 0.05$), NC8-pSIP409-pgsA' ($P < 0.01$) and saline ($P < 0.001$) groups at day 42 (Fig. 5), with an increasing trend from days 28 to 35 (Fig. 5). These data suggested that lavage administration of NC8-pSIP409-pgsA'-S-DCpep could increase IgA antibody titers and produce humoral immune responses in mice.

3.6. NC8-pSIP409-pgsA'-S-DCpep induces the expression of cytokine production

Specific cytokines in the MLN cell secretions were measured 3 days after the last immunization using ELISA. The results showed that the concentration of IL-4 in the supernatants of MLN cells cultured from the NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep-treated

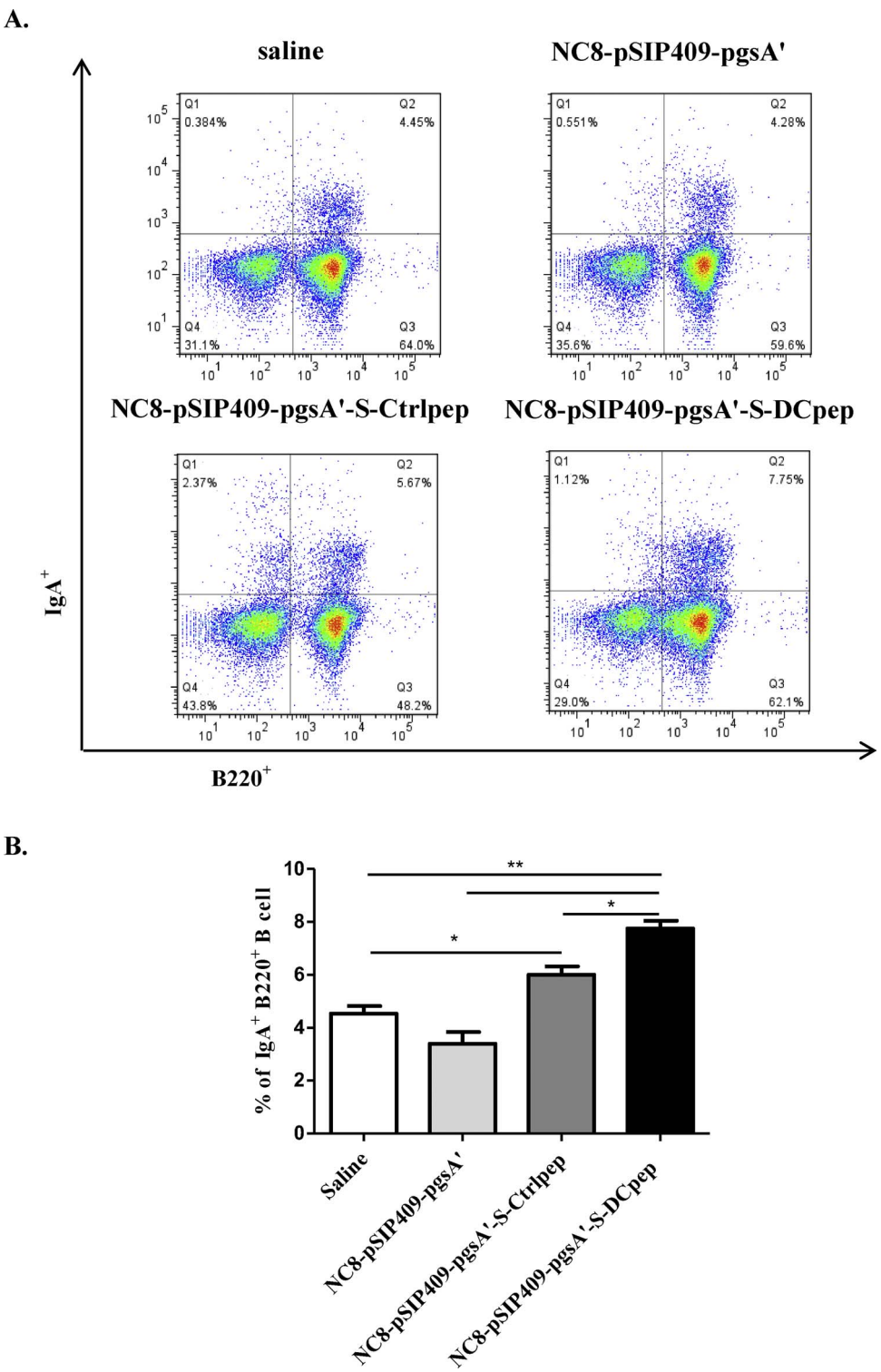

Fig. 3. Detection of activated B cells in PP of immunized mice using flow cytometry. The number of total cells for (A) the quadrants and (B) the bar graphs refer to IgA^+B220^+ cells. The degree of activation of B cells was analyzed by counting the percentage of IgA^+ $B220^+$ expression. Significant differences relative to the saline and NC8-pSIP409-pgsA' controls ($P < 0.05$, $P < 0.01$). Significant differences are denoted by an asterisk (*) between saline, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

mice were significantly increased compared to those from the saline group ($P < 0.01$) (Fig. 6A). Higher level of IL-17 was observed in the group of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep compared to the group of mice orally administered with saline ($P < 0.01$), NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA' groups ($P < 0.05$) (Fig. 6C). Unexpectedly, the level of IFN-γ in the supernatant of MLN cells cultured with the strains expressing S-DCpep was significantly higher in the group of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-Dcpep compare to the group of mice orally administered with saline ($P < 0.01$), NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA' groups ($P < 0.05$) (Fig. 6B).

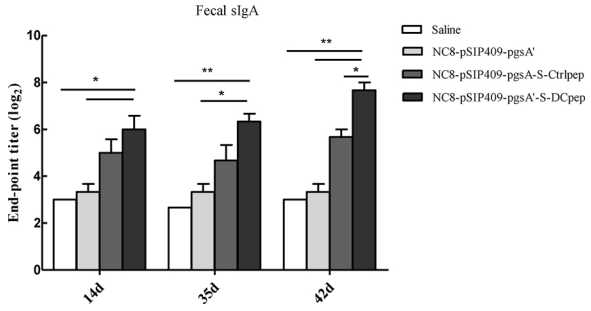

Fig. 4. S-specific sIgA titers levels in mouse feces examined by ELISA se. The S-specific sIgA Ab titers were detected after prime immunization, booster immunization, and the last immunization. End-point titer (log_2) were defined as the highest dilution yielding an absorbance that was two and three times higher than the background for the fecal and serum samples, respectively. Significant differences are denoted by an asterisk (*) between Stroke-physiological saline solution, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

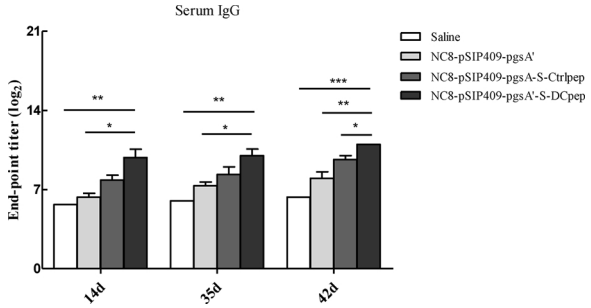

Fig. 5. S-specific IgG titers levels in sera of mice detected by ELISA. The S-specific IgG Ab titers were detected after prime immunization, booster immunization, and the last immunization. End-point titer (log_2) were defined as the highest dilution yielding an absorbance that was two and three times higher than the background for the fecal and serum samples, respectively Significant differences are denoted by an asterisk (*) between Stroke-physiological saline solution, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.7. NC8-pSIP409-pgsA'-S-DCpep induced production of serum/fecal-neutralizing antibodies

Specific PEDV antibodies titers in serum and faeces were measured by cell neutralization assays at different time after the first immunization. Results showed that serum and fecal antibodies of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep was higher than that from mice orally administered with and Saline at days 14 ($P < 0.01$) (Fig. 7). The serum antibody of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep was significantly increased compared with NC8-pSIP409-pgsA' at days 35 ($P < 0.01$) (Fig. 7A), however, the fecal antibodies of NC8-pSIP409-pgsA'-S-DCpep was no significant difference at days 35 compared with saline and NC8-pSIP409-pgsA' at days 14 (Fig. 7B). Significant differences were observed in both serum and fecal antibodies of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep compared with the other three group at days 42 (Fig. 7), particularly, difference were also observed in serum and fecal antibodies between the group of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep and NC8-pSIP409-pgsA'-S-Ctrlpep ($P < 0.05$). The results suggesting that recombinant *L. plantarum* NC8-pSIP409-pgsA'-S-DCpep has good PEDV virus-neutralizing activity in humoral and mucosal immunity.

4. Discussion

Porcine epidemic diarrhea (PED) caused by PEDV is a contagious disease which seriously threaten pig production industry (Huang et al., 2013). PED characterized by high morbidity and mortality in piglets was reported in southern China in fall of 2010 (Huang et al., 2013). Furthermore, in April 2013 PED has also spread throughout and to the highest degree destroyed the American pork industry, the homology between the genome of popular strain in American and China is as high as 97%–99%, which has caused immeasurable economic losses (Huang et al., 2013). The continuing outbreak of PED suggests that it is able elude current immunization strategies (Curry et al., 2017). Currently, there is no effective drug for PEDV treatment, although inactivated and live vaccines are the two most commonly used vaccines, Although live and inactivated vaccines are already in widespread use, development of effective, safe and economical oral vaccines are still the trend of future. And researchs of developing oral vaccine to prevent PEDV have achieved effective protection.

L. plantarum is an intestinal predominant flora with immunomodulatory effects that enhance the chemotaxis and phagocytosis of neutrophils, prevent viral infection and maintain intestinal microbial homeostasis (Li et al., 2017a,b). Therefore, *L. plantarum* with probiotic properties has become good exogenous protein vector platform. The anchorin expressed by pgsA gene can help exogenous proteins to locate to the surface of bacterium and display their biological function. Other and our team have achieved good protection using pgsA to display exogenous antigens, hence, using pgsA to display exogenous antigens during vaccine development is a viable strategy.

The feature of localized intestinal infection of PEDV determined that the immunization and prevention is difficult, hence, intestinal mucosal immunity plays an important role in pigs except suckling piglets (Temeeyasen et al., 2017). Resistance to PEDV infection often relies on IgA secreted by mucosa, which requires the development of vaccines that can effectively increase intestinal immunity. Contemporary strategies in vaccination primarily focus on using different vectors to express the identified antigenic regions. Antigens delivered by different routes might stimulate the intestinal mucosal immune system to achieve immunoprophylaxis effect. The use of probiotics to express antigens and proliferate them in the intestinal of host can promote the digestion of livestock and poultry; meanwhile, the protective antigen expressed can stimulate the intestinal mucosal immune system to produce protective effect.

As a favorable mucosal immunization vaccine vector, *L. plantarum* can also be used as a natural adjuvant in the mucosal microenvironment and attenuate inflammatory response to enhance the body immunity (Yu et al., 2015). Morever, vaccines constructed by DCpep could provide effective protection (Yang et al., 2017a,b). Consequently, we constructed fusion protein by the immunogenic S-protein of PEDV and the induced DCpep and expressed it on the surface of *L. plantarum*, which could activate DCs to initiate mucosal and humoral immune responses against microbial infection via secreting antibodies to clear the antigens. Results showed that the DCpep fusion group had better immune protection compared with other groups.

DCs are the foremost antigen presenting cells (APCs) and play an important role in inherent and adaptive immunity (Sato et al., 2017). $CD11c^+$ is a surface marker of DCs, and $CD80^+$ and $CD40^+$ are subsets of $CD11c^+$. There was a difference in expression of $CD11c^+CD40^+$ between the group of mice orally immunized with recombinant NC8-pSIP409-pgsA'-S-DCpep and saline group but not NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA' groups, however, the MEI of group NC8-pSIP409-pgsA'-S-DCpep was still higher than the above two groups (Fig. 2A). The expression of the $CD11c^+CD80^+$ in NC8-pSIP409-pgsA'-S-DCpep group was significantly higher than the control group and the NC8-pSIP409-pgsA' group in the LP of small intestine (Fig. 2B). In this study, the expression of $CD11c^+CD86^+$ in DCs was no significant difference between NC8-pSIP409-pgsA'-S-DCpep and saline group, which should also increased according to previous studies. This difference in results might be due to different host strains and different immunization programs. However, $CD11c^+CD86^+$ did not affect the biological function of $CD11c^+CD80^+$ and $CD11c^+CD40^+$ DCs, which could also identify $CD40^+$, $CD80^+$, $CD86^+$ and other co-stimulatory molecules,

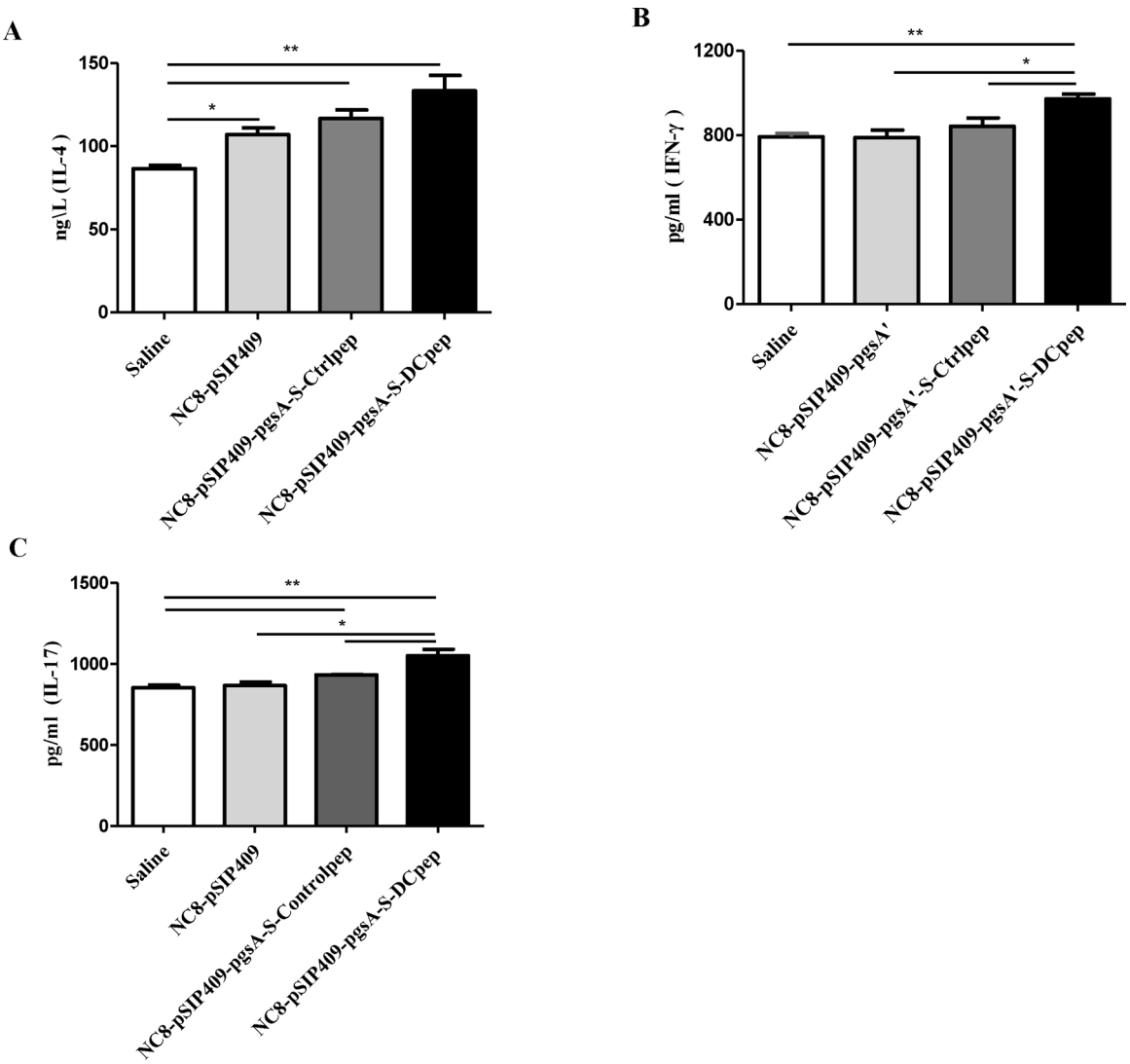

Fig. 6. Secretion levels of S-specific cytokines (A) IL-4, (B) IFN-γ and (C) IL-17 in MLN detected by ELISA. Significant differences are denoted by an asterisk (*) between Stroke-physiological saline solution, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

present antigen to activated T cells or effector T cells. Meanwhile, $CD80^+$ and $CD86^+$ of activated B cells could combine with CD28 expressed on the surface of T cells, which involved in co-stimulatory signal transduction. In addition, the results of S-specific IgG in serum and S-specific sIgA in feces showed that *L. plantarum* strains expressing S protein could improve the secretion of IgG and sIgA and further enhance immune function, particularly for NC8-pSIP409-pgsA'-S-DCpep (Figs. 4 and 5).

Several studies showed that IgA secreted by mucosal lamina propria could combine with mucus and cover on the surface of epithelial cells which inhibit the adhesion of microorganisms to epithelial cells and neutralization of toxins produced by microorganisms or enzymes. SIgA agglutinates and incapacitates pathogens by which it inhibits pathogen adhesion to the mucosal surface and easily cleared in the secretions. Therefore, sIgA disenables pathogens interact with epithelial cell receptors and inhibits the assembly of viral particles within the host cell cytoplasm (Kurashima and Kiyono, 2017; Motegi et al., 2000). Our study suggesting that the anti-S mucosal sIgA responses of recombinant *Lactobacillus* NC8-pSIP409-pgsA'-S-Dcpep group significantly increased. Following four indicators are used to evaluate the effectiveness of a vaccine in mucosal system: connecting closely with lymphoid tissue in mucous membrane; the existence of scattered lymphoid tissues and lymphoid organs with a certain structure; DCs use a special mechanism to uptake antigen; and the presence of many activated and memory lymphocytes in the absence of an infection (Chung et al., 2017; Faria and Reis, 2017). Currently, many investigators are focused on the development of mucosal vaccines. Our study demonstrated that the recombinant *L. plantarum* NC8-pSIP409-pgsA'-S-DCpep could increase the activation of B cells and further improve the level of humoral immunity (Fig. 3). Orally administration of recombinant NC8-pSIP409-pgsA'-S-DCpep in mice boosted the number and frequency of IgA^+B220^+ B cells compared with the NC8-pSIP409-pgsA' and NC8-pSIP409-pgsA'-S-Ctrlpep groups. Similarly, we also detected anti-S systemic IgG in the blood, The results showed that S-DCpep could effectively initiate a mucosal immune response and humoral immunity. Subsequently, *L. plantarum* expressed S-DCpep fusion protein could enhance the secretion of mucosal antibodies against pathogens (Mohamadzadeh et al., 2009; Shi et al., 2016; Yang et al., 2017a,b, 2016). In the construction of the recombinant strain NC8-pSIP409-pgsA'-S-Dcpep, pgsA has been used as an anchorin for its stability and safety in clinical use. Besides, pgsA has a wide range of applications for surface expression on some types of *Lactobacillus* compared with other anchorins. Therefore, we used pgsA as a surface display element for applying to surfaced of *L. plantarum*. A recombinant vaccine constructed by S-protein expressed on the surface of *L. plantarum* and DCpep fusion protein can provide protection since PEDV is primarily replicated in the small intestine. The

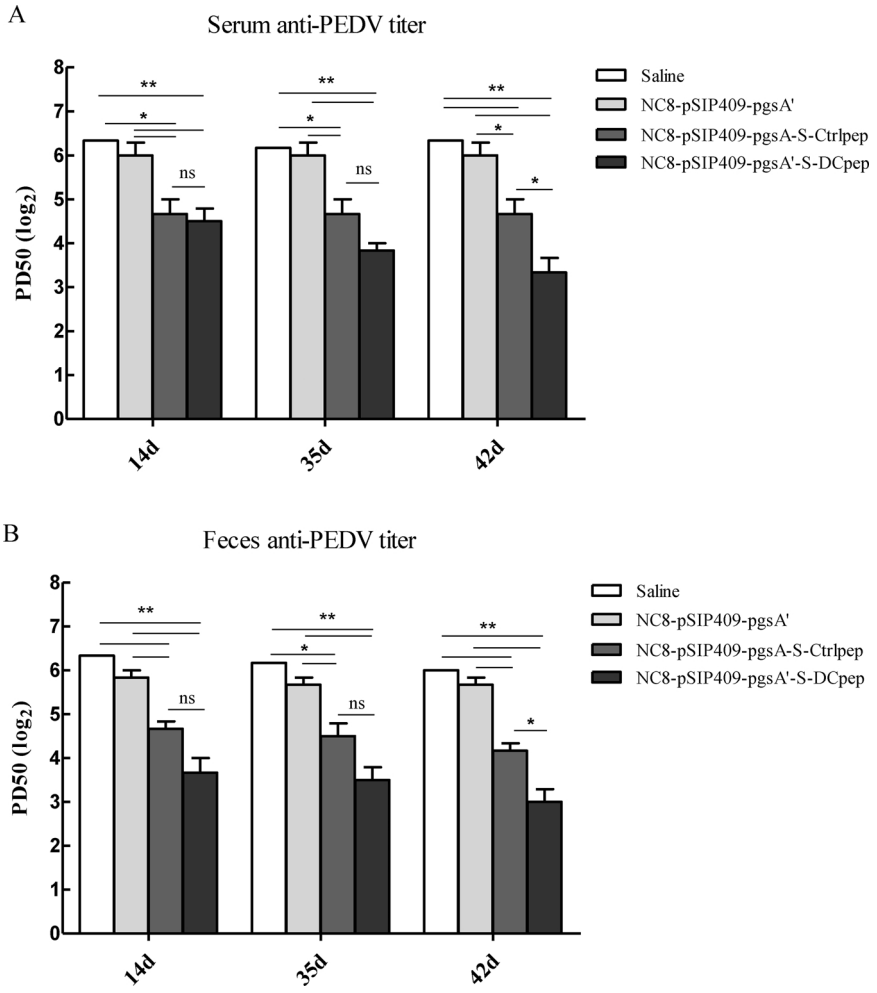

Fig. 7. NC8-pSIP409-pgsA'-S-DCpep induced mice to produce serum(A)/feces(B)-neutralizing antibodies against PEDV. PD50 (Median protective dose refers to the dose of serum or vaccine that protects half (50%) of the experimental animals).Significant differences are denoted by an asterisk (*) between Stroke-physiological saline solution, NC8-pSIP409-pgsA', NC8-pSIP409-pgsA'-S-Ctrlpep and NC8-pSIP409-pgsA'-S-DCpep controls (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

results of western blot, FACS and confocal microscopy analyses suggested that S protein was expressed on the surface of *L. plantarum*.

Compared with traditional intramuscular injection, gavage administration allows antigens to be presented through the gastrointestinal mucosa and obtain better immunological response, which is a simple, convenient and ideal immunization strategy. Several genetically engineered bacteria (*Salmonella, Escherichia coli, Lactobacillus*) could colonize in the intestines after inoculation that were regarded as promising oral vaccine vectors (Garcia-Fruitos, 2012). In addition, peptidoglycan expressed on the surface of *L. plantarum*, could function as a natural immune adjuvant and be utilized to design a novel vaccine (Dieye et al., 2001). Previous studies have demonstrated that *L. plantarum* could act as a mucosal delivery system to induce immune response and tolerance (Tauer et al., 2014). The distinctive feature of the immune response triggered by oral mucosal immunization is the production of specific secretory IgA. Antigens were taken up by M cells after oral inoculation, then dendritic cells and T cell subsets were activated, cytokines and chemokine were released and MHCI and MHCII were expressed (Kurashima and Kiyono, 2017). Finally the activation of B cells, the expression of specific integrin, phenotype transformation (especially to IgA type conversion) are observed (Perdigon et al., 2002).

Th1 and Th2 responses induced by recombinant *L. plantarum* were evaluated via cell-specific cytokines analysis. Th1 cell response can helpcytotoxic T cell differentiation mediate cellular immune responses, secrete IFN-γ and activate macrophages, which enhance the ability of cells to kill phagocytized pathogens (Maldonado-Lopez and Moser, 2001). Besides, the primary function of Th2 cells is to stimulate B cell proliferation and produce antibodies, which is related to humoral immunity (Mosmann and Coffman, 1989). Secretion of IL-4 increased the ability of monocytes differentiate into macrophages (Lee et al., 2009). IL-17 can increase the ability of costimulatory molecules, MHC, antigens and T lymphocytes to stimulate antigen peptide delivery and promote DC maturation (Lee et al., 2009). Therefore, we analyzed cytokine system IFN-γ, IL-4, and IL-17 of Th1/Th2/Th17 cells secreted by the MLN of the immunized animals after treatment with recombinant bacteria. Our results showed that NC8-pSIP409-pgsA'-S-DCpep could significantly increase cytokine secretion and stimulate Th1 and Th2 cells and Th17 cell response, maintain the balance of Th1, Th2 immune response. The focus of our study was Th1 and Th2 immune responses induced by NC8-pSIP409-pgsA'-S-DCpep, which also stimulated the activation of Th17 cells (Fig. 6). Taken together, recombinant *L. plantarum* expressed S-DCpep could improve Th1/Th2/Th17 immune response in BALB/c mice and induce immune responses, such as the differentiation of B cells and DCs maturation.

In this study, we detected PEDV-specific neutralizing antibody in serum and fecal obtained from the group of mice orally administrated with *L. plantarum* expressed PEDV-S protein on the surface, which could significantly inhibit the formation of plaque on Vero cells. The results indicated that systemic immune response induced by recombinant *Lactobacillus* provided an immunoprotective effect against the virus as shown in (Fig. 7). The constructed recombinant *L. plantarum* might contribute to the development of novel vaccines against PEDV

challenge.

In conclusion, our study demonstrates that NC8-pSIP409-pgsA'-S-DCpep can modulate mucosal and humoral immunity and produce specific antibodies against S-protein. Next, we will study the protective effect of recombinant bacteria against PEDV challenge in piglet.

Acknowledgments

The National Key Research and Development Program of China (2017YFD0501000). This work was supported by the National High Tech R&D Program of China (863 program) (2013AA102806), National Natural Science Foundation of China (31672528, 31272541, 31602092), Science and Technology Development Program of Jilin Province (20160519011JH, 20170204034NY), Special Funds for Industrial Innovation of Jilin Province (2016C063), "Thirteen Five-year Plan" for Sci & Tech Research Program of Jilin Education Department of P.R. China [grant number JJKH20170318KJ] and the Doctoral Project sponsored by the Scientific Research Foundation of Jilin Agricultural University of China (201601).

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